MATERNAL-FETAL COCAINE METABOLISM, DISTRIBUTION, AND IMMUNOTOXICOLOGY

By

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Cocaine use by women during pregnancy has prompted interest in cocaine distribution to the mother and fetus and in long term effects of cocaine on fetal development. Conservative estimates indicate that 11-15% of all newborns have been exposed to cocaine during gestation. Hypotheses: the fetus, given unique routes of exposure in utero and immature metabolism, is exposed to higher concentrations of cocaine than the mother during maternal cocaine use in pregnancy; the ingestion of cocaine with alcohol further increases cocaine concentration in both mother and fetus; and cocaine and metabolites have immunotoxicological effects thereby making the neonate more susceptible to opportunistic infections.

Amniotic fluid, cord blood, and neonatal urine were obtained at delivery from women who revealed cocaine use during pregnancy. Cocaine and metabolites were extracted from body fluids using solid-phase extraction and quantified using high performance liquid chromatography and/or gas chromatography-mass spectrometry. Results of human and animal studies indicate that the fetus is exposed to high cocaine concentrations in utero and eliminates the drug more slowly than the mother. In mice, ethanol cotreatment resulted in a fivefold increase in peak cocaine serum concentrations and a sixfold increase in peak cocaine concentrations in the brain. Peak cocaine concentrations were reached faster in ethanol treated mice and rats. These observations suggest that ethanol can alter the pharmacokinetics of cocaine, which could partially account for reports of increased toxicity of the drug when combined with alcohol. Cocaine has multiple actions on several neurotransmitters many of which have a direct effect on immune function. The immunotoxicologic effects of cocaine are therefore of interest. No studies have been published examining the effect of cocaine metabolites on the immune system. Proliferative effects of cocaine and cocaine metabolites benzoylecgonine, norcocaine, and cocaethylene on B lymphocytes were examined using the IM9 human cell line. Results suggest that cocaine has an inhibitory effect on T-cells while having a stimulatory action on B-cells. This effect is similar to that produced by the human immunodeficiency virus. Cord blood mononuclear cells isolated from both cocaine exposed and nonexposed neonates showed similar responsiveness to mitogenic challenge.

CHAPTER 1
BACKGROUND AND SIGNIFICANCE

Cocaine is one of the most widely abused illicit drugs in the United States. An estimated 50 million Americans (25% of the population) have used cocaine and 5-6 million use it regularly [1,2]. The most recent population estimates on the use of cocaine were reported by the National Institute on Drug Abuse and the Substance Abuse and Mental Health Services Administration in the 1992 "National Household Survey on Drug Abuse." Results indicated an estimated 5 million Americans used cocaine in the previous year with the majority of users reporting "crack" cocaine use [3]. As the price of cocaine has dropped in the last 20 years, the number of users has risen significantly and the use pattern has changed from recreational experimentation to abuse. It is estimated that 15-30% of all cocaine users are women of childbearing age [1,3]. Recently, more attention has been directed to this population and some hospitals perform routine screening of newborn's urine for drugs of abuse. From such screening studies, it has been reported that 11-15% of all pregnant women have used cocaine during pregnancy [4-7]. However, there appears to be significant geographical variation in prevalence. One recent study from a Chicago hospital yielded a prevalence rate of 37% of very-low birthweight babies testing positive for cocaine and/or cocaine

metabolites [8], while 18% of newborns tested positive in a Boston survey and 2-3% in a Rhode Island survey [9]. In a survey of 36 hospitals from different geographical regions across the country, the overall prevalence rate was 11% with reports ranging from 0.4-27% [6]. The effect of cocaine use by the mother during pregnancy has been associated with medical complications in the newborn. Of particular concern at this time is the effect of cocaine on immune function as over 75% of all perinatally acquired human immunodeficiency virus (HIV) infections are associated with intravenous drug use by the mother or her sexual partner [9].

Pharmacological and Physiological Effects of Cocaine

Cocaine has multiple actions affecting dopaminergic, cholinergic, serotonergic, and noradrenergic receptor sites. Cocaine enhances the experience of pleasure with increased alertness, sexuality, and energy, and decreased anxiety and social inhibition. However, while the use of low doses of cocaine is reported to enhance feelings of pleasure, the chronic use of high doses of cocaine may lead to irritability, aggression, paranoia, anorexia, and psychosis. The effects of cocaine on the central nervous system (CNS) are well characterized and demonstrate a biphasic pattern of intense stimulation followed by depression. Cortical stimulation is manifested by euphoria, restlessness and excitability. However, none of the known neurochemical actions of cocaine singly provide a full explanation of how the drug produces euphoria. In the CNS, cocaine blocks the reuptake of dopamine at the dopamine transporter in the synaptic cleft thereby producing an accumulation of dopamine at postsynaptic receptor sites (Figure 1-1). This, in turn, increases

neurotransmission in the mesolimbic and mesocortical dopaminergic pathways in the brain. These pathways are termed the "reward center" of the brain and function in producing the euphoria associated with cocaine use.

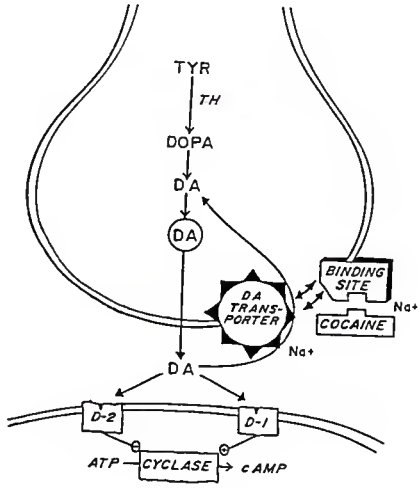

Figure 1-1: Effects of cocaine at dopaminergic synapses [10].

It is not clear, however, whether the euphoria is a direct effect of cocaine on dopaminergic neurons or a combination effect due to simultaneous actions on other neurotransmitters, like serotonin [10-12]. By blocking the reuptake of dopamine with chronic cocaine use, the tissue stores of this neurotransmitter are gradually depleted and this results in compensatory upregulation and supersensitivity of dopamine receptors [1,11]. The blockade of dopamine reuptake is associated with the intense reinforcing stimulus of cocaine [13,14]. Cocaine also blocks the

reuptake, turnover, and synthesis of serotonin (5HT) and this mechanism may be involved in the euphoric action of cocaine as well as the onset of tolerance and effects on sleep, appetite, and aggression [1,14].

Cocaine also blocks the reuptake of catecholamines, primarily norepinephrine (NE), at adrenergic nerve endings potentiating sympathetically mediated vasoconstriction, tachycardia, hyperglycemia, and hypertension [1,4,15]. Initially, in the brain, the levels of NE are elevated; however, cocaine activates the presynaptic alpha-2 receptor which, in turn, produces feedback inhibition of NE. As with dopamine receptors, chronic cocaine use results in compensatory upregulation of adrenergic beta receptors in the CNS.

The toxic effects of cocaine have been the subject of a great deal of research in recent years and several complications associated with cocaine abuse have been identified. Pyrexia is a prominent feature of cocaine poisoning. Heat retention occurs due to vasoconstriction, increased muscle activity, and direct effects on the heat regulating center of the diencephalon [15]. Death by respiratory collapse has occurred immediately after IV administration of cocaine and pulmonary edema is a common clinical finding at autopsy following cocaine overdose. The use of crack cocaine has been associated with characteristic pulmonary injury known as "crack lung" which is characterized by inflammatory cell infiltration and alveolar hemorrhage [16]. Oral and nasal ingestion have resulted in death after a symptom free period of one hour followed by the onset of generalized seizures [17]. Cerebrovascular accidents, including seizures, intracerebral hemorrhage,

subarachnoid hemorrhage and cerebrovascular infarction, have also been reported. The mechanism of hemorrhage is thought to be associated with cocaine-induced hypertension causing the rupture of a pre-existing lesion [15,18]. Cocaine and metabolites are also reported to be potent vasoconstrictors of cerebrovascular arterioles [19,20]. The accumulation of catecholamines predisposes the myocardium to arrhythmias which may compromise cardiac output, in some cases resulting in myocardial infarction and ischemia due to the myocardial increased demand for oxygen. Due to the vasoconstrictive action of cocaine, the myocardium is not able to utilize compensatory autoregulatory mechanisms involving vasodilation in response to the increased oxygen demand. Cocaine toxicity has also been implicated in sudden arrhythmic death, aortic rupture, contraction band necrosis, cardiomyopathy, and myocarditis [15, 21-30]. Intestinal ischemia may result from cocaine induced catecholamine stimulation of alpha receptors in mesenteric vasculature, causing intense vasoconstriction and reduced blood flow [15,30]. Death from cocaine overdose usually results in 1-5 hours, and the number of cocaine related emergency room visits have tripled in the last 10 years.

Additionally, cocaine also serves as a local anesthetic and this was one of the earliest uses of cocaine in the United States. Cocaine has been used as a local anesthetic in dental, nasal, and ophthalmic surgery. The pharmacologic action of cocaine (as well as other local anesthetics) is the blockade of sodium channels in the membranes of neuronal cell bodies, neuronal axons, and cardiac muscle [31,32]. Sodium channel blockade prevents the propagation of action potentials to

neighboring cells and the nerve conducts fewer impulses. While unionized cocaine penetrates the membrane faster, the cationic form predominates at physiological pH and is the active form of the drug at the receptor site. When compared to other local anesthetics, cocaine has a unique effect in that it also acts as a vasoconstrictor at the site of application. This prolongs the local anesthetic effect and reduces bleeding. Other local anesthetics are often administered with a vasoconstrictor, such as epinephrine, to produce a similar effect. Since cardiac muscle cells also contain sodium channels, these are also blocked by cocaine. This disrupts normal cardiac pacemaker activity and conduction, which when combined with the blockade of norepinephrine reuptake, can produce the arrhythmia and hypertension discussed previously.

Finally, studies in rodents and non-human primates indicate cocaine also affects normal endocrine function and alters secretion of anterior pituitary hormones via its action on central dopamine systems [33-37]. Pituitary prolactin secretion is under tonic inhibitory regulation by dopamine (dopamine is known as prolactin inhibitory factor (PIF)). Dopaminergic neurons in the tuberofundibular pathway (connecting the arcuate nuclei with the hypothalamus and posterior pituitary) are responsible for prolactin release from the pituitary. In human studies, chronic cocaine use has been associated with persistent hyperprolactinemia in cocaine users [38-40]. A controlled study with 8 male human cocaine users demonstrated that hyperprolactinemia was associated with chronic cocaine use; however, there was no difference in the pulse frequency of prolactin release. This indicated that

the hyperprolactinemia may have resulted from the effect of cocaine on dopaminergic inhibition of basal prolactin secretion [34]. A follow-up study by this group with 42 patients undergoing treatment for cocaine addiction found that while hyperprolactinemia was not common, its presence was a poor prognostic sign for abstinence from cocaine use [41]. Research in this area is on-going, especially evaluation of the role of serotonin on hypothalamic-pituitary-adrenal axis dysregulation.

Cocaine Administration and Pharmacokinetics

Cocaine is a naturally occurring alkaloid found in the leaves of the South American shrub *Erythroxylon coca*. The leaves are estimated to contain up to 2% cocaine by weight and are harvested at 3-4 years old [4,42]. South American natives have chewed the coca leaf for centuries for its stimulant effects, and it was introduced to the United States in 1885 for use both as a local anesthetic and in some over-the-counter products, including wine and Coca-Cola [1,4,13]. In the early 1900s, as the abuse potential of cocaine became evident, laws were passed to restrict its use. By World War II, all states had laws prohibiting cocaine use for other than medicinal use as a local anesthetic. Presently, cocaine is placed in Schedule 2 of the Controlled Drug Act following guidelines stipulated by the federal government. Drugs placed in Schedule 2 have an accepted medical use, but also have high abuse potential. Cocaine powder, or cocaine hydrochloride, is extracted from the coca leaves using acids and an organic solvent like gasoline. This form of cocaine is a water-soluble salt which can be administered by nasal insufflation

or by intravenous injection of an aqueous solution. However, since 1985, free-base cocaine, in the form of "crack" cocaine, has become the most widely used form of the drug. "Crack" cocaine is an impure free-base manufactured from cocaine powder by mixing with ammonia, baking soda, and warm water. The free base precipitates in the alkaline water and the water is then boiled away leaving hard chunks of the drug [43,44]. "Crack" is not water-soluble but is stable at temperatures required for pyrolysis and is administered by vaporization via smoking in a water pipe. Crack cocaine can be obtained illicitly for as little as $10 and the rapid, intense euphoria produced makes it highly reinforcing [45].

The pharmacokinetics of cocaine is dependent on the route of administration. Natives of the Andes and Amazon chewed coca leaves with ash thereby forming a coca paste. The coca paste was either held in the mouth for long periods of time and gradually swallowed or it was mixed with tobacco and smoked. The chewing of paste does not produce euphoria, but does provide mild stimulation and enhancement of endurance. The chewing of powdered coca leaves which contained 17-48mg of cocaine was reported to produce peak plasma concentrations of 10-150µg/L within 0.4-2 hours in 6 volunteers [46]. The chewing of 50g of coca leaves in a 3 hour period produced plasma cocaine concentrations of 150-450ng/ml in native Peruvians [47] and similar cocaine levels were reached much faster when coca paste was smoked [48].

Cocaine is absorbed from mucous membranes and the gastrointestinal tract, although oral ingestion is not common. Gastrointestinal absorption is slow and

bioavailability of cocaine is estimated to be 30% by this route [4]. Gastrointestinal absorption is slow since cocaine (with a pKa of 8.6) would be ionized in the stomach (pH 2.0) and would perhaps be more readily absorbed from the more alkaline small intestine (pH 6.0-8.0). One study using human volunteers measured plasma cocaine concentrations after oral administration of a dose of 2mg cocaine hydrochloride per kilogram body weight in a gelatin capsule [49]. For comparison, the same dose was also administered topically to the nasal mucosa. Results indicated that after oral administration, cocaine was not detected in plasma for 30 minutes. Peak plasma concentrations of 104-424ng/ml were reached in 50-90 minutes and then declined over the next 4-5 hours and the half-life was 0.9 hours. Results of intranasal application demonstrated detectable plasma cocaine concentrations by 15 minutes and peak concentrations of 61-408ng/ml at 60-120 minutes with a half-life of 1.3 hours. Subjective measures of "high" by the subjects were reached faster in the intranasal applications [49].

Nasal insufflation of cocaine hydrochloride was once the predominant route of drug administration by users [50]. After topical application of a cocaine dose of 1.5mg/kg body weight to the nasal mucosa of 13 human patients, the absorption half-life was about 12 minutes and peak plasma levels of 100-500ng/ml were achieved in 15-60 minutes. Plasma cocaine concentrations then decreased gradually over the next 3-5 hours, although cocaine was still detectable on swabs from the nasal mucosa three hours after the application [51]. In a study designed to parallel the illicit intranasal use of cocaine, 10 human volunteers were instructed

to inhale 100mg of a mixture of cocaine hydrochloride powder (16-96mg) and lactose through a 5.0 cm straw [52]. On alternate days, subjects were given 16-32mg of cocaine hydrochloride intravenously in 1ml of saline. Plasma cocaine concentrations were measured after dosing. After nasal insufflation, peak plasma cocaine concentrations of 53-206ng/ml were reached in 30-60 minutes; however, plasma cocaine concentrations persisted for a longer period of time than subjective drug effects reported by the subjects. After intravenous administration, peak plasma cocaine concentrations of 200-300ng/ml were reached in 5 minutes and physiological and subjective drug effects paralleled declining cocaine concentrations [52]. Jeffcoat et al [53] used radiolabeled cocaine to examine disposition after nasal insufflation by human volunteers. A dose of 106mg of [4-^{3}H]-cocaine (equivalent to 94.6mg of cocaine) was insufflated through a glass tube. The average bioavailability was 80%. The absorption half-life was 11.7 minutes and the elimination half-life was 80 minutes with the mean peak plasma cocaine concentration of 220ng/ml reached by 45 minutes.

Intravenous injection of cocaine and smoking free-base cocaine produce similar pharmacokinetic profiles and both produce an intense euphoria within 1 minute of administration. Intravenously injected cocaine has been reported to reach the brain in 16 seconds; however, due to efficient respiratory exchange, smoked free-base cocaine reaches the brain faster (in 8 seconds), but has a shorter duration of action [4]. Jeffcoat et al [53] also administered radiolabeled cocaine to human volunteers by intravenous injection and smoking. A dose of 23mg of [4-^{3}H]-

cocaine (equivalent to 20.5mg of cocaine) was dissolved in 3 ml of saline and then injected into an antecubital vein over a 1 minute period. The mean peak plasma cocaine concentration of 180 ng/ml was reached by 5 minutes with the distribution half-life reported to be 11 minutes and the elimination half-life 78 minutes [53]. For administration via smoking, a dose of 50 mg of [4-^{3}H] cocaine free base was placed in a glass pipe and the pipe was heated to 250°C in a hot oil bath. Subjects were instructed to inhale vapors for 10 seconds (holding the inhaled vapor for 15 seconds) at 30 second intervals for 5 minutes. The absorption half-life was 1.1 minutes and the mean peak plasma concentration of 203ng/ml was reached by 6 minutes [53]. The average bioavailability was 57%; however, a previous study demonstrated that most undecomposed cocaine reaches the circulation and the lower bioavailability is related to decomposition before inhalation [54]. The inhalation efficiency of "crack" pyrolysis has been measured as 73±9% and 62±11% at 170°C and 220°C respectively [55].

Plasma cocaine concentrations after multiple dosing were also reported following intravenous and pulmonary administration of cocaine to human subjects by Isenschmid et al [56]. Subjects were given two separate doses of cocaine spaced 14 minutes apart by either intravenous injection (16mg or 32mg cocaine) or by smoking (25mg or 50mg cocaine). Plasma samples were analyzed for cocaine and metabolite concentrations and were found to be dose dependent. Peak cocaine concentrations ranged from 210ng/ml for 25mg cocaine via smoking to 470ng/ml for 32mg cocaine via intravenous injection. Results indicate that the

half-life of cocaine was 38-39 minutes regardless of dose or route of administration. To simulate the binge cocaine use of a typical user, some subjects were given 5-7 doses of cocaine in 90 minutes using a mixture of intravenous injection and smoking. The maximum cocaine concentration measured was 1200ng/ml from an individual who received 16mg of cocaine by IV bolus and then smoked 50mg cocaine 6 separate times over 90 minutes. In subjects smoking 50mg cocaine 6 separate times over 90 minutes, the average plasma cocaine concentration achieved was 890ng/ml [56].

The pharmacokinetics of cocaine have been studied by many investigators with most reporting the half-life to be 43-60 minutes [52,53,56-58]. The volume of distribution of cocaine is small and reported values range from 1.2-2.7L/kg [4,18,53,59]. When plasma levels of cocaine and urinary excretion data were plotted together, the decline of the excretion rate plot paralleled that of the decline in plasma indicating cocaine follows a linear pharmacokinetic model [60]. Garrett and Seyda [61] found the plasma protein binding of cocaine to be less than 10% and not a significant pharmacokinetic variable. However, this report is challenged by Edwards and Bowles [62] who report that cocaine is highly bound to the serum proteins albumin and alpha-1-acid glycoprotein. Support that this protein binding is not a significant factor, however, comes from placental transfer studies which found that while cocaine was 50-90% bound to serum proteins, diffusion across the placenta was still rapid and cocaine binding to umbilical cord serum was significantly less than to maternal serum [63].

Cocaine Metabolism

Only 1-5% of a dose of cocaine is excreted unchanged in the urine [4,60]. The remainder is metabolized by several pathways to water soluble metabolites which are excreted in the urine (Figure 1-2):

(1) Cocaine is hydrolyzed by plasma and liver cholinesterases to ecgonine methyl ester; in addition, non-enzymatic hydrolysis and hydrolysis by liver carboxylesterases to benzoylecgonine also occurs. Ecgonine methyl ester is primarily a urinary metabolite and is usually detected in the blood only in post-mortem cases [64]. These pathways account for 70-90% of cocaine metabolism in adults.

(2) The second pathway is via N-demethylation by mixed function oxidase to norcocaine, a physiologically active cocaine metabolite [65]. Human cytochrome P450 3A is the oxygenase responsible for the oxidative metabolism of cocaine.

(3) When cocaine is used simultaneously with ethanol, a transesterification reaction occurs in the liver (catalyzed by hepatic carboxylesterase) to form cocaethylene [66-68]. Cocaethylene is an active metabolite retaining cocaine's pharmacologic and toxicologic properties and has a half-life of more than twice that of the parent compound.

(4) Other, minor metabolites have been identified in the blood and urine of cocaine users including, ecgonine, ecgonidine, norecgonine methyl ester, and anhydroecgonine [69,70].

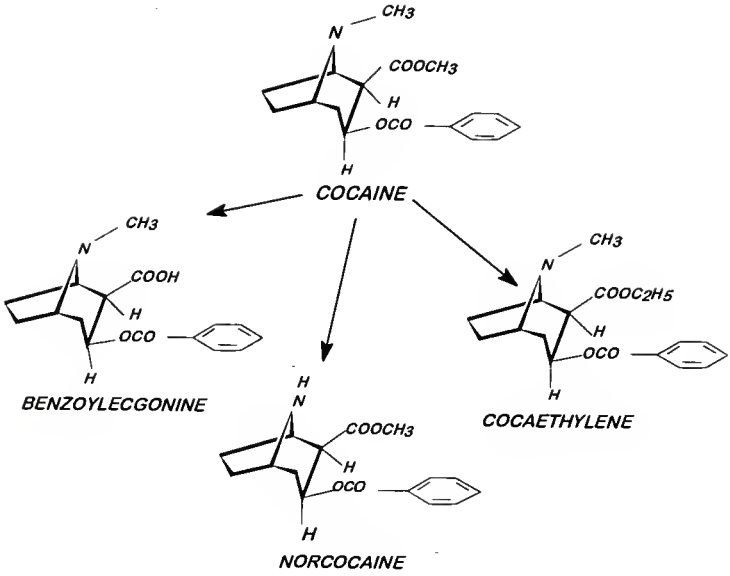

Figure 1-2: Routes of metabolism of cocaine *in vivo*.

Benzoylecgonine and ecgonine methyl ester are water soluble metabolites readily excreted in the urine and can be detected for 24-72 hours after a cocaine dose. Acidification of the urine, in combination with diuretics, can increase renal excretion of cocaine. Renal excretion of cocaine in the neonate may be slow as the glomerular filtration rate is only 30-40% that of an adult and is directly related to gestational age. In addition, the rate of tubular secretion in neonates is only 20-30% that of an adult and the renal blood flow velocity is significantly lower [31,71]. Only 2-4% of a cocaine dose is excreted in the feces and bile.

As mentioned earlier, plasma and liver cholinesterases metabolize cocaine. Therefore, any factor affecting esterase activity could have a significant effect on cocaine metabolism. It has been reported that pregnant women, fetuses, and people with liver disease have lower cholinesterase activity thus potentially increasing the risk of cocaine toxicity in these groups [4,15]. In addition, due to genetic polymorphisms, 1-13% of North Americans are considered to have little or no cholinesterase activity [72]. The formation of norcocaine by N-demethylation was higher in subjects with lower cholinesterase activity [65]. Ecobichon and Stephens [73] were able to demonstrate that plasma pseudocholinesterase activity in the neonate was 50-60% of that in the adult. Detectable levels of pseudo-cholinesterase were observed at 28 weeks of gestation and activity increased rapidly until 1 year of age when the neonatal levels reached those of adults. Their observations suggested that premature neonates would have significantly different rates of hydrolysis of ester-type drugs [73]. The activities of the fetal drug

metabolizing enzymes are only 50-70% that found in adults and the half-lives of various drugs in the neonate are significantly longer than the half-lives in adults [31].

Aspects of drug metabolism and monoamine transport by the placenta must also be considered in this discussion. The placenta essentially serves as a sieve for the passage of drugs from the maternal circulation to the fetus. Lipophilic drugs readily cross the placental barrier and only drugs of very high molecular weight or drugs that are highly ionized are unable to cross the membranes. Cocaine and several cocaine metabolites readily cross the placenta via passive diffusion [63,74]. However, water soluble metabolites like benzoylecgonine do not diffuse well. When formed in the fetus, these metabolites will tend to accumulate on the fetal side due to ionization of the drugs since the pH of fetal blood is slightly lower than the pH of maternal blood.

The placenta also serves as a site of oxidative metabolism for some drugs (i.e. ethanol and pentobarbital) including hydroxylation, demethylation, and dealkylation reactions [31]. The placenta has considerable amounts of smooth endoplasmic reticulum and expresses several families of cytochrome P450. The cytochrome P450 family CYP3A, including 3A3,4, and 7 is responsible for a portion of cocaine metabolism. CYP3A4 and 7 are expressed in fetal liver and are also expressed in placental tissue [75-77]. However, in studies by Schenker et al [74] and Krishna et al [63] with perfused human placenta there was no evidence of placental metabolism of cocaine. Conversely, Roe et al [72], using human placental microsomes, showed a 20% decrease in cocaine concentration over a 130 minute

in vitro incubation period. In addition, using human placental villus tissue homogenates, Ahmed et al [78] were able to identify a protein binding site with high specificity for cocaine. Acetylcholinesterase has also been identified in human placental villus tissue homogenates indicating the placenta may have the capacity to metabolize cocaine; however, there is evidence that the placental acetylcholinesterase is different both genetically and functionally from plasma cholinesterase [79].

Human placenta expresses transporters for both serotonin [80] and norepinephrine [81]. These transporters are present on the brush border membranes which are in contact with the maternal blood. While the purpose of these placental transporters in fetal development has not been elucidated, in the CNS, the disruption of monoamine transport by cocaine produces significant physiological responses. The presence of these transporters may indicate the placenta may actually serve as a primary target organ for cocaine and, therefore, as an important mediator of the fetal effects of maternal cocaine use. Work with the placental serotonin transporter has shown that cocaine is a potent competitive inhibitor of the transporter [80]. It is speculated that serotonin, a vasoconstrictor, may be involved in regulation of the uteroplacental circulation; thus, the action of cocaine at this transporter would result in increased concentrations of serotonin and vasoconstriction of the placental vasculature [80].

Materno- Fetal Circulation and Pharmacokinetics

During gestation, the placenta serves as the system for fetal respiratory gas exchange, nutrient supply, and removal of metabolic wastes. The fetal lungs are filled with fluid until just prior to birth. The maternal and fetal circulatory systems are separate; however, there is diffusion between the circulations via the placenta. Oxygenated blood enters the placenta via the maternal uterine and ovarian arteries and the maternal spiral arteries, where the oxygen is exchanged for carbon dioxide with the fetal blood. Drugs used by the mother during pregnancy also diffuse from the maternal circulation through this process. The placento-fetal circulation consists of the umbilical cord, which has 2 arteries and 1 vein. Since gas and nutrient exchange is handled by the placenta during gestation, circulatory adaptations are present in the fetus which are then adjusted on delivery to the circulation present throughout postnatal life. These adaptations include the ductus venosus, foramen ovale, and ductus arteriosus. Oxygenated blood enters the fetus via the umbilical vein and a substantial amount is shunted through the ductus venosus to the inferior vena cava and right atrium; however, some blood enters the fetal liver through the portal vein. Blood is then shunted from the right atrium through 2 pathways: (1) through the foramen ovale, to the left atrium, and then to the ascending aorta. This pathway provides the circulation to the fetal heart, brain, and upper extremities; (2) to the right ventricle and then to the pulmonary circulation (less than 10% due to the high pulmonary resistance) and through the ductus arteriosus to the descending aorta and lower body. Blood then returns to the placenta via the umbilical arteries.

After birth, once the umbilical cord has been clamped, the ductus venosus closes, the respiratory center is activated, and blood flow increases through the pulmonary veins. The foramen ovale closes as left atrial pressure rises over right atrial pressure and the ductus arteriosus flow reverses and then constricts due to the high oxygen tension of the blood. If the ductus arteriosus remains patent after birth, it must be either induced to close by administration of indomethacin (which promotes closure by inhibition of prostaglandin synthesis) or, as a last resort, it must be surgically ligated. A recent study has found that prenatal cocaine exposure is associated with indomethacin failure and subsequent surgical ligation of the patent ductus arteriosus (R. F. Covert, University of Chicago, personal communication).

Complete evaluations of human maternal-fetal cocaine pharmacokinetic relationships have proven difficult to measure due to the complex technical issues confronted. Factors that must be considered in such pharmacokinetic determinations would include maternal cocaine absorption, distribution, and elimination, fetal distribution and elimination, placental transfer (bi-directional), placental disposition, and amniotic fluid disposition. Several compartmental models have been proposed to model maternal-fetal drug kinetics. Szeto [82] proposed a two-compartment model, one compartment representing the mother and the second representing the fetus. Drug elimination from both mother and fetus would be considered. Another possible compartmental model would include a multicompartment arrangement with compartments for the placenta and amniotic cavity (Figure 1-3).

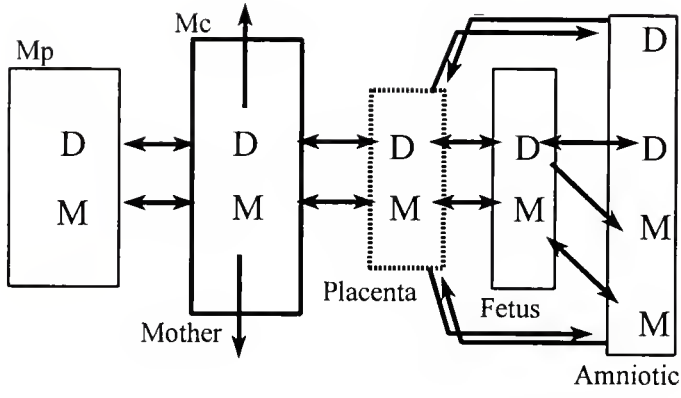

Figure 1-3: Proposed maternal-fetal pharmacokinetic model. (Modified from Devane [83]).

Physiological models have also been proposed [83,84]. These models consider drug disposition to the tissues and organs of the body as a function of time and can model altered physiological states. Luecke et al [84] have developed a sophisticated, computer driven, physiologically based model for human pregnancy that considers the rapid growth that occurs during pregnancy. However, since available data from human pregnancy are generally limited to single time-point measurements taken at delivery and since cocaine doses are only obtained from patient self-report, most cocaine pharmacokinetic determinations have utilized animal models. Sheep are often the animal model of choice given the relatively large fetal size and the ability to cannulate fetal vessels and withdraw blood for

measurement of drug levels. This may, however, not be a good model for cocaine metabolism and pharmacokinetics.

Drug Distribution to the Fetus

Maternal drug use during pregnancy is of great concern due to the rapid passage of drugs across the placenta discussed earlier in this chapter. Therefore, it would also be useful to determine to what extent the fetus is exposed to drugs ingested by the mother. Many studies have observed that the fetal concentration of a drug is lower than the maternal concentration. Drugs studied have included methadone, morphine, meperidine, lidocaine, aspirin, and dilantin [82].

Information on fetal concentrations of cocaine is somewhat inconclusive. Devane and colleagues [85] reported that the time of sample collection was an important consideration when comparing maternal and fetal plasma cocaine concentrations. Pregnant ewes were administered an IV bolus of cocaine in saline at doses of 0.5-4.0mg/kg and both maternal and fetal plasma cocaine concentrations were measured over time. Results showed higher maternal cocaine concentrations until 4 minutes after the cocaine dose at which time the maternal and fetal concentrations were of similar magnitude. The area-under-the-curve (AUC) for the maternal samples was substantially greater than the fetal [85]. A separate study also utilizing pregnant ewes found that fetal blood concentrations of cocaine observed 5 minutes after maternal infusion were approximately 12% of the maternal level [86].

In pregnant rats, following a single intraperitoneal dose of cocaine (30mg/kg), cocaine was distributed throughout the fetus with the highest concentrations found in the placenta, followed by the fetal liver, maternal heart, fetal brain, and maternal brain and plasma [87]. Spear et al [88] reported fetal plasma cocaine concentrations, following subcutaneous dosing in pregnant rats, to be ½ to ⅓ of those of the dams. It was suggested that the difference could be explained by lower plasma protein binding in the fetus. Dow-Edwards [89] administered a single dose of cocaine (60mg/kg) by intragastric gavage to pregnant rats on day 22 of gestation and cocaine concentrations were measured at time points for 90 minutes. Cocaine plasma levels peaked at 15 minutes in both dams and fetuses and the mean peak maternal concentration was 1.8 times higher than the fetal; however, maternal cocaine levels decreased faster than fetal levels, and from 30-90 minutes fetal levels were higher than maternal levels. Cocaine studies in pregnant mice showed significantly lower cocaine concentrations in fetal tissues following a single cocaine dose [90]. However, chronic cocaine dosing in rabbits demonstrated fetal cocaine concentrations near or above maternal levels [91]. In that study, pregnant New Zealand White rabbits received 6 doses of cocaine (0.5mg/kg IV) between days 22-28 of gestation. Mothers were sacrificed on day 29 and the maternal and fetal tissue cocaine concentrations were measured. Fetal brain mean cocaine concentration was 20% higher than mean maternal brain concentration, but mean maternal liver cocaine concentration was higher [91].

Chronic dosing in the pregnant guinea pig (chosen for its metabolic similarity to humans) demonstrated a similar effect. In that study, pregnant Dunkin-Harley guinea pigs were given a daily subcutaneous dose of cocaine (6mg/kg) from days 50-59 of gestation. Starting 1 hour after the last injection, sampling was begun as follows: amniotic fluid collected from around each fetus, cord blood collected from the umbilical veins, brains removed, maternal blood collected by cardiac puncture, and maternal urine collected if possible. Maternal and fetal plasma cocaine concentrations were not significantly different and the cocaine brain to plasma ratios were also not significantly different. Higher benzoylecgonine concentrations were found in maternal plasma, but fetal plasma contained higher concentrations of benzoylnorecgonine. However, amniotic fluid cocaine concentrations were 3 times that found in either the maternal or fetal plasma [92].

Controlled pregnancy studies are difficult to implement with non-human primates due to their tendency to abort the fetus if it is surgically removed, cannulated, and returned to the womb. In the only published report found, 4 pregnant baboons were studied at days 155-160 of gestation (term is 184 days). Catheters were placed in the femoral arteries and veins of both the mothers and the fetuses and catheters were also placed in the amniotic sac. Mother baboons were administered a single dose of cocaine (2mg/kg IV) over a 2 minute period and then blood and amniotic fluid concentration-time profiles were measured for 24 hours. Serum cocaine concentrations were observed to decline in a biphasic manner in both mother baboons and their fetuses; however, while the mean AUC for the fetal

samples was lower than the mean maternal AUC, the elimination half-life in fetuses was 25% longer [93].

Effects of Gestational Cocaine Exposure on the Neonate

Given the documented physiological and toxicological effects of cocaine use and the evidence that cocaine readily crosses the placental barrier, the effects of maternal cocaine use in pregnancy on the fetus and newborn have been examined. Documented labor and delivery complications associated with maternal cocaine use have included spontaneous abortion [15,94], stillbirth [94,95], preterm labor and delivery [96-100], premature rupture of the membranes [101,102], and abruptio placentae [5,97,103,104]. The pathophysiological mechanism believed to be responsible for the documented obstetric complications is the accumulation of catecholamines in the periphery produced by cocaine. Accumulation of catecholamines, in turn, produces vasoconstriction in the maternal and placental circulation and increased uterine contractility which leads to placental abruption [15,30,104]. In addition, intrauterine growth retardation has also been reported to be significantly higher in cocaine users [102,105]. Placental insufficiency and decreased blood flow produced by chronic cocaine use may be a major factor on intrauterine growth regulation. Experimental evidence in pregnant sheep administered intravenous doses of cocaine have demonstrated maternal and fetal hypertension, decreased uterine blood flow, increased uterine vascular resistance, elevated levels of serum catecholamines, and fetal hypoxemia [106-109]. Using conscious fetal lambs, Covert et al [109] also reported reduced fetal cerebral blood

flow following cocaine administration (acute IV bolus) to the ewe, but increased cerebral blood flow following cocaethylene administration. However, in work involving acute IV continuous infusions of cocaine to either pregnant ewes or fetal lambs, it has been reported that cocaine increased fetal cerebral blood flow [110]. In this work, cocaine administered to the ewe decreased fetal oxygen tension and oxygen content; however, cerebral and myocardial blood flows were increased which compensated for the effects of the fetal hypoxemia. Infusion directly to the fetus did not alter cerebral blood flow [110].

On the surface, it would appear these independent reports yield opposing results; however, there are very significant differences in the experimental protocols which could account for these observations: (1) cocaine administration: acute IV bolus (1mg/kg) versus acute continuous infusions lasting 30 minutes (0.3mg/kg); (2) time of blood flow measurements: Covert et al [109] began blood flow measurement immediately upon dosing while Burchfield et al [110] controlled for behavioral state. Cocaine administration to either the pregnant ewe or directly to fetal lambs has been reported to disrupt REM sleep in the fetal lambs [108,111]. Thus, Burchfield et al [110] monitored the behavioral state of the fetus and began blood flow measurements only once the fetus had returned to REM sleep after the infusion was stopped. Usually, a recovery time of 1.5-2 hours was required for the fetus to resume REM sleep. REM periods were chosen for blood flow monitoring because cerebral blood flow is as much as 35% higher during REM and REM sleep is a metabolically active state [112].

Fetal hypoxemia, secondary to the placental vasoconstriction induced by cocaine, may also be a contributing factor to the higher incidence of meconium-stained amniotic fluid seen in pregnant women who have used cocaine during pregnancy [113-116]. It has been suggested that meconium-stained amniotic fluid is indicative of fetal stress. Meconium staining is associated with fetal cardiac disturbances and aspiration of meconium by the fetus can result in respiratory distress and infection.

Maternal cocaine use in pregnancy is also associated with adverse affects in the neonate. These problems have included lower gestational age, lower birth weight, length, or head circumference, a higher incidence of intrauterine growth retardation and/or small for gestational age infants, fetal hypertension and ventricular tachycardia, longer newborn hospital stay, and lower Apgar scores (a measure of newborn health) with poorer general outcome [6,94,99-101,114,117]. Other medical problems reportedly associated with maternal cocaine use have included retinopathy and ophthalmic abnormalities [118] and perinatal seizures and cerebral infarction [8,119]. The risk of sudden infant death syndrome is reported to be 3-7 times higher in newborns exposed to cocaine during pregnancy than in the general population [5,96,120,121]. Case reports also indicate an increased risk of necrotizing enterocolitis (NEC) in cocaine exposed newborns [122,123]. NEC is a serious illness, characterized by ischemic necrosis of the intestinal tract complicated by mixed-organism infection, with a high mortality rate [124]. The

illness is generally seen in small, preterm infants and develops after the onset of feeding and would be exacerbated by the vasoconstriction produced by cocaine.

The evidence that prenatal cocaine exposure is associated with a higher rate of congenital malformations is weak. Bingol et al [95] studied 50 cocaine using women and 110 polydrug abusers, and noted a higher malformation rate in the cocaine group. However, the teratogenic information reviewed to date indicates that prenatal cocaine use is not a significant teratogen and the only consistent teratogenic findings have been those of the genitourinary tract [102,125,126].

Despite limited reports that infants and toddlers exposed to cocaine *in utero* are more jittery, irritable, and inconsolable, and less organized and interactive [119], the long-term behavioral and neurological consequences of prenatal cocaine exposure are unknown. In rodents and primates, gestational cocaine exposure was associated with decreases in learning and memory [9]. The National Institute on Drug Abuse is currently funding longitudinal studies in Seattle, Pittsburgh, Miami, and rural Florida where cocaine exposed newborns are followed for 3-5 years to study their neurobehavioral outcomes [9]. Results of these studies will provide evidence for more definitive statements regarding the effect of prenatal cocaine exposure on neurobehavioral progress.

Finally, prenatal cocaine exposure is also associated with increased fetal and newborn deaths [96,127-129]. Fetal deaths in many cases were attributed to placental abruption, respiratory distress, and prematurity. A majority of the mothers in fatal cases had received no prenatal care. Two additional cases of newborn

death associated with cocaine using mothers, but not prenatal exposure, also warrant attention as they are not isolated occurrences [9]. One infant who had been born cocaine positive later died from dehydration and malnourishment following maternal neglect and a second newborn died from acute cocaine intoxication resulting from cocaine laced baby formula. Both cases were classified as homicides [130].

Immunotoxicology of Cocaine

In addition to the toxic effects of cocaine previously discussed, evidence exists to suggest cocaine has a marked effect on the immune system. The pharmacologic effect of cocaine on modulation of monoamine transport would indicate that cocaine may have a significant effect on the regulation of the hypothalamic-pituitary-adrenal axis and therefore, a direct effect on the immune system. If cocaine is toxic to the developing fetal immune system, maternal cocaine use in pregnancy could potentially increase the risk of the newborn acquiring opportunistic infections.

The immune system is characterized by mechanisms of innate and acquired immunity that protect the body from attack by foreign substances. Innate immunity is characterized by mechanisms always present in the body to act as barriers to infection: mucous membranes, coughing, pH, fever, interferon, granulocytes, and macrophages. Acquired immunity is the mechanism by which the body responds to foreign substances (called antigens) after a previous exposure. Acquired immunity functions through the activation of lymphocytes to respond to the antigen.

The cell types involved in acquired immunity include T and B lymphocytes and macrophages. Human lymphocytes possess binding sites for dopamine as well as adrenergic, cholinergic, and opioergic binding sites [131]. These dopamine binding sites resembled neuronal uptake sites and dopamine binding at this site was inhibited by cocaine and other biogenic amine inhibitors [131]. In a separate study, cocaine and other monoamine uptake inhibitors were found to inhibit *in vitro* mitogen stimulated B and T lymphocyte proliferation in rats [132]. These reports indicate cocaine may have the potential to modulate the immune response directly through effects on lymphocytes as well as indirectly through hypothalmic-pitutitary-adrenal axis neuroendocrine pathways [133].

The purpose of the developing fetal immune system is to produce functionally mature B and T lymphocytes. T cells are responsible for cellular immunity and recognize foreign antigens in association with a major histocompatability (MHC) bearing accessory cell (macrophage) that has processed, and presents, the antigen to the T cell. For example, resting T cells will recognize cells that are infected with intracellular organisms like a virus or bacteria. The virus or bacteria is endocytosed by the accessory cell and then the antigen surface marker proteins and MHC molecules are expressed on the cell surface. The resting T cell is then activated by its interaction with the accessory cell and the activated T cells are then stimulated to produce and release lymphokines. B cells constitute humoral immunity and are responsible for synthesis and secretion of immunoglobulins (IgG, IgA, IgM, IgE, IgD). These immunoglobulins are able to bind

to circulating free antigens, including bacteria and toxins. The B cell response is, however, dependent on cooperative interactions with activated T cells. Activated T cells release lymphokines which are known as interleukins (IL) because of their effects on other lymphocytes as well as their action as autocrine growth factors. These interleukins include IL2, 4, and 5 and IL-4 and 5 activate the B cell response [134]. Additional evidence demonstrates that B cell differentiation may be stimulated by direct cell to cell contact between B cells and T cells, specifically CD4+ T helper cells, not just via lymphokines [135].

B and T lymphoctye development is initiated during the first trimester and matures during the second trimester [136]. A mature B or T cell is defined as one that expresses a surface receptor marker for recognizing specific antigens. Important T cell markers include CD4 or T helper cells and CD8 or T suppressor/cytotoxic cells. T cells mature and differentiate in the thymus and the thymus develops early in gestation. Lymphocytes are recognizable by week 10 of gestation and by week 17, the distribution of T cell surface markers is essentially that of the adult. By week 15, fetal T cells will respond to the mitogen phytohemagglutinin (PHA). T cell maturation occurs only during fetal development and for a short time after birth and the T cells then populate the secondary lymphoid organs (spleen and lymph nodes). B cells are first observed in the fetal liver by 9-10 weeks of gestation. B cells are then observed soon after in the fetal bone marrow, spleen, and blood. At this time in fetal development, B cells only express IgM and synthesize no other immunoglobulins. Later in gestation, maternal IgG

crosses the placenta and provides the fetus and neonate with some resistance to infection until the newborn is able to synthesize immunoglobulins. B cells reach the numerical adult number in the cord blood by week 15 of gestation and, by the second trimester, B cell generation shifts to the bone marrow [136]. Therefore, any substance taken during gestation that alters this maturation process could severely compromise the immune system of the newborn.

Although several studies have investigated the effects of cocaine on human and animal immune functions, the relationship remains unclear. Studies evaluating the effect of cocaine on cellular immune function have indicated that the drug causes suppression of mitogenic responses in human and mouse T lymphocytes [137]. These studies, however, involved incubation of the cells with relatively high concentrations of cocaine (12.5-300µg/ml). A complicating factor of many published studies with cultured lymphocytes has been that the dose levels far exceed blood plasma levels found in human cocaine users [51,53]. Cocaine has also been shown to suppress proliferation of phytohemagglutinin (PHA)-activated human peripheral blood lymphocytes at lower drug concentrations (0.9-12.0µM) [138]. Previous work with cultured mouse splenocytes demonstrated that cocaine induced suppression of PHA-activated cells was a biphasic process [139]. More significantly, cocaine (at concentrations observed in human cocaine abusers) was demonstrated *in vitro* to augment human T-lymphocyte proliferation stimulated by the anti-CD3 antibody [140]. This was designed to more accurately mimic the *in vivo* stimulation of T-cells via the T-cell receptor complex than is PHA activation.

Additional work demonstrated that the effect of cocaine stimulated T-lymphocyte proliferation was modulated by alterations in calcium mobilization and IL-2 production [141]. Recently, it was reported that cocaine impairs early activation of CD4+ T-cells [142]. The specific sub-population of CD4+ T-cells affected was the population carrying a surface marker that targets the cells to migrate to the lymph nodes. The *in vivo* effect of this impaired activation would potentially lead to greater risk for the development of opportunistic infections.

In one of the more promising reports to date on the immunotoxicology of cocaine, Ruiz et al [143] found alterations in specific T cell subpopulations in cocaine-intoxicated individuals. Total numbers of natural killer cells, T and B lymphocytes, and T cell sub-sets including memory CD8 T cells, activated T cells, T helper cells, and T helper/inducer cells were counted using flow cytometry combined with immunofluorescence staining targeted to surface marker antigens. Results demonstrated a reduction in the total number of T cells in cocaine-intoxicated individuals which was primarily a result of a decreased percentage of CD4+ T helper cells. In addition, CD8+ "memory" cells were also reduced. However, there was also a preferential stimulation of activated T cells in these individuals. These results indicate that in cocaine-intoxicated individuals there may be a dysfunctional immune response. In response to an immunological challenge, a larger proportion of T cells would be activated but they would not evolve into memory cells [143]. The effect on the immunocompetence of human fetal T-cell

sub-populations would be of particular concern due to the chronic exposure of the fetus to cocaine *in utero*.

Cocaine has been shown to stimulate both natural killer cell activity and natural killer cell numbers in human volunteers who received an intravenous infusion of cocaine [144] and also in cocaine-intoxicated patients admitted to a hospital emergency room [143]. However, an *in vitro* study indicated that cocaine had no effect on natural killer cell activity and no effect on cytotoxic T lymphocytic activity [145]. Thus, cocaine may have differential effects *in vivo* on recruitment versus activation of immune cell subpopulations.

Studies of humoral immunity have produced less definitive information on the immunomodulatory effects of cocaine. Klein et al [139] reported that the lymphoproliferative response to a B cell mitogen was not affected by cocaine at a concentration which suppressed T cell proliferation. In another study, rats injected with cocaine for a 10 day period were shown to have elevated B cell responses to helper T cell independent and dependent antigens. However, in this same study, *in vitro* exposure of lymphocytes to cocaine had no effect on B cell proliferation [146]. Previous work by Havas et al [147] in mice found that, even at lethal doses of cocaine, the B cell response was elevated.

There is limited evidence to support indirect effects of cocaine on human B cells. Elevated serum levels of IgG have been reported in IV drug users; however, when human peripheral blood mononuclear cells (PBMC's) were cultured with pokeweed mitogen (a B cell activator) and cocaine there was no effect of the

cocaine on IgG synthesis when compared to drug free controls [148]. This indicates cocaine, and other IV drugs, may induce B cell activation through indirect mechanisms; however, the elevated IgG levels may be an effect of the presence of more infectious agents seen in the IV drug using population [148]. In a related report in the rat, cocaine (1mg/kg) was administered IV to the jugular vein 2 times per day for 12 consecutive days. Serum immunoglobulin levels were measured 24 hours after the last cocaine dose and compared to controls. Results demonstrated significant increases in IgG levels in the cocaine treatment group and smaller, although significant, decreases in serum IgA [149].

Finally, the potential role of cocaine as a co-factor in human immunodeficiency virus (HIV) infection and in the development and progression of acquired immunodeficiency syndrome (AIDS) has been raised [150-152]. The role of cocaine as a co-factor in perinatal HIV transmission has also been raised [153]. Early indications are that HIV infection associated with cocaine users is a result of intravenous drug use or sexual contact with an intravenous drug user [150,154]. In addition, female cocaine users also demonstrate increased incidence of sexual transmitted diseases attributed to a more sexual promiscuous lifestyle [151,155]. However, after adjusting for sexual promiscuity and prostitution, the rates of infection were still higher in cocaine using women indicating involvement of some other factor [155]. A recent study in Baltimore of 140 IV drug users found that 43% of females and 57% of males were HIV positive. When correlated to drug usage, the group with the highest infection rate was cocaine-preferring females with 58%

HIV positive [156]. Cocaine has also been shown to potentiate the replication of the HIV virus in human peripheral blood mononuclear cells in *in vitro* culture [157,158].

Cocaine and Alcohol Interactions

Many addicts report the concomitant use of ethanol and cocaine and report a better "high" when both drugs are used [68,159]. In fact, Gorelick [160] reports that clinical and epidemiological data indicate the co-abuse of these substances is more common than might be expected and that the 1988 NIDA household survey found that 80% of the cocaine users also reported ethanol use. An earlier survey found that 30% of cocaine users drank ethanol every time they used cocaine. One estimate indicates that as many as twelve million people may use these drugs in combination in any one year [68]. If the combination of cocaine and ethanol were to increase the concentration of cocaine in the systemic circulation and the brain, the toxicity experienced by the fetus could be exacerbated.

Ingestion of cocaine and ethanol leads to the *in vivo* formation of the active metabolite cocaethylene (also known as ethylcocaine or ethylbenzoylecgonine). This metabolite has been detected in the urine of cocaine users also testing positive for ethanol [159,161]. *In vitro* studies indicate the formation of CE occurs via a transesterification reaction in the liver catalyzed by hepatic carboxylesterase forming a 2-carboxyethyl ester in place of the 2-carboxymethyl ester of cocaine [66,162]. This enzyme is also reported to catalyze the conversion of cocaine to benzoylecgonine. Controlled studies *in vivo* in rats and mice have found that serum benzoylecgonine concentrations are lower with ethanol pretreatment indicating

ethanol inhibits the formation of benzoylecgonine while simultaneously catalyzing the formation of CE [162,163]. The enzymatic formation of CE has been demonstrated utilizing human liver extracts and human, mice, and rat liver microsomal homogenates [66,163,164]. *In vitro* incubation of cocaine and ethanol with homogenates of human kidney, brain, liver, lung, and placenta show that only liver homogenates produce cocaethylene [165].

Cocaethylene retains the pharmacologic and toxicologic properties of cocaine yet has a half-life of 2 hours compared to the 45-60 minute half-life of cocaine [68]. This metabolite has high affinity for the dopamine transporter in the CNS and may enhance the euphoria experienced by cocaine users due to the blockade of dopamine reuptake [166]. However, the effect on modulation of the serotonin system was significantly less than cocaine indicating CE is targeted to dopaminergic function [167]. An enhanced and prolonged euphoria was reported by subjects administered both cocaine and ethanol. The plasma cocaine and norcocaine concentrations were also significantly higher in the acutely intoxicated individuals as compared to those receiving only cocaine [67]. In primates, CE was equipotent to cocaine in maintaining self-administration [168].

Because of the high rate of the co-abuse of ethanol and cocaine, one major effect of the combination has been an increase in cocaine related hospital admissions and sudden death [68,169,170]. It has been reported that the co-abuse of these drugs increases the risk of cocaine-related sudden death 18-fold [164]. Significant levels of CE have been measured in postmortem blood and brain

samples from cocaine related deaths [166]. In one study, urine and blood, collected from 15 patients admitted to the hospital for trauma (motor vehicle accidents, gunshot or stab wounds), were analyzed for cocaine and cocaine metabolites. Cocaethylene was detected in the plasma of 13 of the 15 patients and some samples had only cocaethylene detected, no cocaine [170]. Cocaethylene has also been detected in the urine of newborn babies [8,169]. This cocaine metabolite is believed to mediate cocaine related toxicity and lethality [171,172] exhibiting an LD_{50} in mice of 60.7mg/kg versus 93.0mg/kg for cocaine. Cocaethylene has also been found to be toxic to liver hepatocytes [173] and ethanol has been found to potentiate the hepatotoxicity of cocaine [174-176].

Although the cocaine/ethanol metabolite cocaethylene will be used in immune function studies, the role of ethanol alone will not be studied as it has been extensively reviewed [177-179].

Analytical Methods for Detection of Gestational Cocaine Exposure

Due to the potential for adverse effects of gestational cocaine exposure, efficient and sensitive methods of detection are needed to identify those newborns exposed. The most common analytical method for detection of drugs of abuse is a urine screen using an immunoassay technique, followed by solid-phase extraction of the drug from the biological matrix and structural confirmation with gas chromatography-mass spectrometry. Immunoassay is a rapid, relatively inexpensive screening procedure which is designed primarily to eliminate drug free samples. However, immunoassay is fairly non-specific and any sample which tests

positive for a particular drug must be confirmed by a more specific chromatographic analysis. Immunoassay is based on the reaction of an antibody with the drug for which the sample is being tested, ultimately producing a measurable response. In the case of cocaine, the immunoassay test involves the reaction of an antibody with benzoylecgonine, the principle hydrolysis product of cocaine in adult urine. This antibody is much less cross-reactive towards cocaine or other metabolites such as norcocaine or EME. Immunoassay methods commonly used include enzyme multiplied immunoassay (EMIT), fluorescence polarization immunoassay (FPIA), and cloned enzyme donor immunoassay (CEDIA) which are homogeneous assays requiring no separation step of bound drug from unbound drug. Radioimmunoassay (RIA) is also used and is a non-homogeneous assay requiring separation of bound from unbound drug. For urine testing, the legal and commercially established immunoassay cut-off limit for benzoylecgonine is generally 300ng/ml.

Chromatographic techniques used for analysis of cocaine and metabolites have included both gas chromatography-mass spectrometry (GC-MS) and high performance liquid chromatography (HPLC). GC-MS provides the highest degree of specificity and is the accepted "gold standard" method of analysis for cocaine, benzoylecgonine, and EME [56,69,180-185]. The main disadvantage to GC-MS is that compound derivatization is routinely required prior to analysis. The use of HPLC is becoming more common as the advent of diode-array and multiwavelength detectors has improved the selectivity of the method giving ultraviolet (UV)

absorption profiles and derivative spectral data for each peak in the chromatogram. Very sensitive HPLC methods have been developed for cocaine detection and quantitation in a variety of biological matrices [18,92,186-189]. The main disadvantages of HPLC are that EME is not detectable using UV detection and that matrix interference is more common than that seen with GC-MS.

Urinalysis of newborn urine has limitations. In most cases it only indicates recent use (within 48-72 hours) of the drug by the mother. This sample may be highly inaccurate in newborns due to the elapsed time from presentation of the mother for labor, delivery, and subsequent urine collection in the nursery . However, when the urine of 70 newborns born to cocaine using women was sampled every 8 hours for 6 days after delivery, benzoylecgonine was detected in some newborns up to 120 hours (mean 80 hours) after delivery [97]. In the case of detection of cocaine exposure in newborns, it has been suggested that meconium, the first feces of the baby, is a better indicator of cocaine exposure [190,191]. It is hypothesized that drugs in the maternal-fetal circulation may accumulate in the meconium from the time it is first produced at about week 18 of gestation. Since the initial reports by Ostrea et al [190], other investigators have confirmed that meconium was a better biological matrix than urine for detection of gestational cocaine exposure [192-195]. However, a recent report employing structured drug use interviews found that meconium testing did not improve detection of cocaine use occurring in early to mid-gestation; in fact, overall results found testing of urine (maternal or fetal) and meconium produced equivalent results when the same analytical methods

were used. Cocaine use had to have occurred in the three weeks prior to delivery to elicit a positive cocaine test using meconium [196].

Amniotic fluid may also be a good biological matrix for detection of prenatal cocaine exposure. Reports from cocaine studies using guinea pigs, sheep, and humans indicate amniotic fluid often contains high concentrations of cocaine and/or cocaine metabolites [92,109,129,197-199]. Since the concentrations in amniotic fluid are often much higher than in maternal urine or serum, cocaine and metabolites may accumulate in this medium. Since the fetus is exposed to the contents of the amniotic fluid, both orally via swallowing and transdermally, during the gestational period, the determination of the presence and concentration of compounds in this fluid may be significant as it would prolong fetal exposure to the toxic effects of cocaine. Using esophageal ligation in fetal lambs, Mahone [198] demonstrated cocaine and metabolites were still detected in the fetal plasma when cocaine was infused into the amniotic fluid. It has been reported that the fetus swallows between 200-450ml of amniotic fluid per day and urinates as much as 500ml per day into the amniotic cavity [200]. Since the fetus urinates into the amniotic cavity, the presence of benzoylecgonine in the amniotic fluid is indicative of cocaine metabolism by the fetus since benzoylecgonine does not cross the placenta from mother to fetus [74,109].

The above review of the literature indicates the following: that fetal metabolism and elimination of cocaine differs from that of adults, the concomitant use of alcohol with cocaine may exacerbate the toxic effects of cocaine, and

cocaine has a modulatory effect on the immune system. This research was therefore designed to investigate the following hypotheses.

Hypotheses for this Study

(1) The main hypothesis is that the fetus is exposed to higher concentrations of cocaine than the mother during maternal cocaine use in pregnancy.

(2) The ingestion of cocaine with concurrent ethanol use further increases cocaine concentration. In pregnancy, cocaine concentration is increased in mother and fetus.

(3) Cocaine and its metabolites have an adverse effect on the immune system (*in vitro*).

Specific Aims

1. To develop and validate analytical methods for the analysis of cocaine and cocaine metabolites benzoylecgonine (BZE), norcocaine (NC), and cocaethylene (CE) in biological samples.
2. To characterize maternal/fetal cocaine metabolism and distribution using both human samples and animal models.
3. To characterize cocaine metabolism and distribution with the concurrent use of ethanol using animal models.
4. To examine the effect of cocaine, benzoylecgonine, norcocaine, and cocaethylene on T and B lymphocyte proliferation (*in vitro*).

CHAPTER 2
METHODS

Biological Samples

Human biological fluid and tissue samples from were provided by subjects identified by neonatologists at the University of Illinois Hospital (Chicago, Illinois), the University of Chicago Hospitals (Chicago, Illinois) or at Shands Hospital (Gainesville, Florida). The use of these samples was approved by the Institutional Review Boards at all participating universities. Animal studies were conducted at the University of Florida - Gainesville and approved by the Animal Care Committee.

Amniotic fluid (1-5ml) was collected at the time of membrane rupture, delivery, or amniocentesis. Upon arrival in the nursery, a urine bag was placed on the infant to collect a sample of the first voided urine (1-5ml) and before 24 hours of age. Urine samples were then transferred to a plastic sample container. The newborn's meconium was collected from the diaper and placed in a plastic sample container. All samples were stored frozen at -20°C until analysis. Cord blood (5-20ml) was obtained after delivery and placed in a heparinized blood collection tube. Serum and tissue samples from animal studies were stored at -20°C until analysis.

Specific Aim #1

To develop and validate analytical methods for the analysis of cocaine and cocaine metabolites benzoylecgonine, norcocaine, and cocaethylene.

Basic Materials

Methanol, acetonitrile, and potassium phosphate monobasic (all HPLC grade) were purchased from Fisher (Fair Lawn, NJ). Diethylamine, butylamine, chloroform, isopropanol, sodium hydrogen carbonate, sodium hydroxide, acetic acid, hydrochloric acid, ammonium hydroxide, and tris buffer were reagent grade (Fisher, Fair Lawn, NJ). Lipase was reagent grade (Sigma, St. Louis, MO). Cocaine hydrochloride, benzoylecgonine, and bupivacaine were obtained from Sigma (St. Louis, Mo). Cocaethylene and norcocaine were obtained from the National Institute on Drug Abuse (NIDA, Rockville, MD).

Fluorescence Polarization Immunoassay Screening

Instrumentation, materials, and calibration

An Abbott Laboratories AD_x^{TM} fluorescence polarization immunoassay instrument (Abbott Park, IL) was used for screening of urine, amniotic fluid, and meconium extracts. This technology uses specific antibodies in the reagent systems that react with free drug (benzoylecgonine) in the sample to form an antibody-drug complex. Therefore, when analyzed by this method, a "positive" sample indicated the presence of cocaine metabolite. The reagent kit contained all components needed for the reaction of the antibody with any free drug in the

sample which included: antibody, antigen-tracer, and a pretreatment which prepared the sample for analysis. Kits for this system have been developed by the manufacturer for analysis of either urine or serum. Dilution buffer, reagent kits, calibrators, and controls for benzoylecgonine were purchased from Abbott (Abbott Park, IL).

The AD_x^{TM} was calibrated using 6 calibration solutions prepared in human urine with a benzoylecgonine concentration range of 0-5000ng/ml. Low, medium, and high control solutions of benzoylecgonine were included in all runs for quality control monitoring. All calibrators, controls, and samples were diluted with an equal volume of buffer (pH 7.7) during sample analysis.

Cocaine hydrolysis experiment

Since the AD_x^{TM} screens samples only for the presence of benzoylecgonine, any sample which contained only cocaine would screen "negative". Previous work however indicated high concentrations of the parent drug (cocaine) in meconium and amniotic fluid. This experiment was therefore designed to find the optimal conditions at which cocaine (spiked into urine) was completely hydrolyzed to benzoylecgonine allowing its subsequent detection by immunoassay techniques. Drug free urine was obtained from a volunteer. A 100µg/ml sample of cocaine hydrochloride was prepared by solubilizing 0.01g cocaine hydrochoride in 100ml of drug free human urine. Standards were prepared by dilution of the stock solution with human urine to yield cocaine concentrations of 1000ng/ml and 100ng/ml. Samples were incubated over a range of pH values of 8.1-10.4 (the pH adjusted

using concentrated NaOH), for time periods from 15-60 minutes, in erlenmeyer flasks placed in a warm water bath (50°C). After the incubation period, the pH of the sample was immediately adjusted using 6M HCl to a final pH range of 8.1-10.4 for analysis by AD_x™. Samples were analyzed by AD_x™ and reported values of benzoylecgonine were compared to the known concentrations.

Clinical samples of urine and meconium

Subsequently, a series of actual clinical samples of newborn urine and meconium were screened by both the direct and the hydrolysis method to determine if hydrolysis provided improved detection of gestational cocaine use. Ten samples of urine and 5 samples of meconium were used. One aliquot of the urine sample was analyzed directly by AD_x™. A second aliquot of urine (400µl) was adjusted to pH>9.5 with concentrated NaOH, incubated for 60 minutes at 50°C, and then adjusted to pH 8.0 for analysis by AD_x™. The following procedure was used for meconium analysis by AD_x™: (1) The meconium sample was weighed and placed in a test tube (12X75mm); (2) Either 100% methanol (2ml) was added (for direct analysis) or 3% ammoniacal methanol (2ml) was added (for hydrolysis); (3) The mixture was vortexed and centrifuged for 10 minutes at 1000g; (4) Followed by incubation for 60 minutes at 50°C; (5) The supernatant was then transferred to a clean test tube and evaporated to dryness; (6) The residue was reconstituted in AD_x™ buffer (0.5ml) prior to analysis by AD_x™. Benzoylecgonine concentrations measured using both methods were then compared.

Solid Phase Extraction (SPE) Procedures for Biological Samples

Meconium

Meconium (0.5-1.0g) was extracted by suspension and vortex mixing with 3ml of methanol. After centrifuging for 10 minutes at 1000g, the remaining methanolic supernatant was transferred to a stoppered tube and vortexed with 0.025M potassium phosphate buffer (1ml) at pH 3 and 10µl of internal standard (bupivacaine 100µg/ml). This extract was then applied to a Strong Cation Exchange column (SCX) (Varian, Harbor City, CA.) with a capacity of 1ml, which had been conditioned under vacuum on a Vac Elut manifold (Varian) with methanol (2ml), water (1ml) and 0.25M phosphate buffer (1ml). After application of the sample, the column was air dried for approximately 30 seconds and then washed with phosphate buffer (1ml) and 0.1M acetic acid (0.5ml). The column was again air dried for 30 seconds before eluting the adsorbed drugs with 3% ammoniacal methanol (2ml). The final extract was evaporated to dryness under nitrogen.

Urine

An aliquot of urine (0.5-1ml) was mixed with an equal volume of 0.025M potassium phosphate buffer at pH 3 and also with 10µl of internal standard (bupivacaine 100µg/ml) and then applied directly to an SCX extraction cartridge and extracted as described above for meconium. Extracts were evaporated to dryness under nitrogen.

Whole blood or cord blood

Due to the lipophilic nature of whole blood samples, a more polar extraction column was utilized for the successful extraction of cocaine and metabolites from this matrix. Whole blood or umbilical cord blood (0.5-1ml) was extracted using the following procedure: Bond Elut (Varian, Harbor City, CA.) columns, containing C2 packing material with a capacity of 3ml, were positioned on a Vac Elut vacuum manifold and conditioned with methanol (3ml), followed by 0.1M sodium hydrogen carbonate (3ml) at pH 8.5. The blood sample was diluted with 1ml of carbonate buffer (pH 8.5) and 10µl of internal standard (bupivacaine 100µg/ml) and then applied to the extraction column and allowed to dry for 30 seconds before it was washed with carbonate buffer (3ml), followed by 1ml of 5% v/v methanol in water. The absorbed drugs were then eluted from the column with 6 X 0.250 ml of chloroform : isopropanol (4:1). The extracts were evaporated to dryness under nitrogen.

Amniotic fluid

The viscosity of amniotic fluid required the use of a high flow extraction cartridge as follows: Amniotic Fluid (1-5 ml) was added to phosphate buffer (1-5 ml, pH 6) and 10µl of internal standard (bupivacaine 100µg/ml). The mixture was applied to an X-TracT column (United Chemical Technologies, Horsham, PA), with a capacity of 15ml, previously conditioned with methanol (5ml), water (3ml) and phosphate buffer (3ml) at pH 6. The sample was drawn through under vacuum and the column air dried for 30 seconds. The column was then washed with water

(2ml), 100mM hydrochloric acid (2mL), and methanol (3ml). The column was again allowed to dry before eluting adsorbed compounds with 10ml of a mixture of chloroform : isopropanol : ammonium hydroxide (78:20:2). The eluent was evaporated to dryness under nitrogen.

Brain tissue

Two methods were used to extract drugs from brain tissue. Due to its lipophilic nature, brain tissue must be digested prior to extraction. In method 1, the brain sample was weighed in a sample vial. Then, 3ml of Tris buffer (0.2M) was added to the vial along with 1mg of lipase and 10µl of internal standard (bupivacaine 100µg/ml). The mixture was homogenized using a mechanical homogenizer and then incubated at 60°C for 2.5 hours. The sample was then extracted using Bond Elut (Varian) columns containing C2 packing material and with a capacity of 1ml as described above for whole blood. In method 2, the brain tissue was weighed in a large, conical tube. Then 3ml of 0.025M potassium phosphate buffer at pH 3 and 10µl of internal standard (bupivacaine 100µg/ml) were added. The sample was homogenized using a mechanical homogenizer and centrifuged for 10 minutes at 1500g. The supernatant was then extracted using SCX columns with a 1ml capacity as described above for urine. Extraction recovery was determined using both for comparison.

Serum or plasma

Serum and plasma from humans or animals was extracted following the method described above for urine.

Placenta and liver

Placental and liver tissue were extracted using method 2 described above for brain tissue and using C2 extraction columns with a 1ml capacity.

High Performance Liquid Chromatography (HPLC)

Mobile phase

The initial mobile phase used was that of Browne et al [192] which consisted of 0.025M potassium phosphate buffer : acetonitrile (85:15) containing diethylamine (25ml/L) with the final pH adjusted to 2.9 with concentrated orthophosphoric acid. Mobile phase 2 consisted of 0.025M potassium phosphate buffer (with 2% butylamine) : acetonitrile (78:22) with the final pH adjusted to 3.0. Mobile phase 3 consisted of 0.025M potassium phosphate buffer : acetonitrile : butylamine (78:20:2) with the final pH adjusted to 3.0. Mobile phase 4 consisted of 0.025M potassium phosphate buffer : acetonitrile : butylamine (81:18:1) with the pH adjusted to 3.0 with concentrated orthophosphoric acid. Solvents used were of HPLC grade and were degasssed by bubbling with helium prior to use.

Analytical column

Two analytical columns were utilized throughout this research. Column 1 was a Waters (Marlborough, MA) Novapak C18, 4um, ODS column (15cm x 3.9mm ID). Mobile phase was delivered to this column at 0.5 ml/min. Column 2 was an Alltech (Deerfield, IL) Lichrosorb RP18 10um column (25cm X 4.6mm ID). Mobile phase was delivered to this column at 1.5 ml/min.

Instrumentation

Analysis was performed using two separate systems. System 1 used a Waters (Marlborough, MA) Model 510 pump to deliver mobile phase to the analytical column. A C18 Novapak Guard Pak precolumn (Waters, Milford, MA) was used to protect the analytical column. The sample was injected using a Waters model UK6 universal liquid chromatograph injector equipped with a 50µl sample loop. The detector was a Spectra Physics Focus multiwavelength detector (Thermo Separation Products, Winter Park, FL) with an IBM Personal System/2 data system for acquisition, integration, and processing. The eluent was monitored at 230, 255 and 275nm and full spectra were recorded from 190 to 400nm for each peak. Quantitative analysis was achieved by comparison of peak areas of unknowns with extracted standards. All peak areas were reported as relative peak areas by comparison to the peak area of the internal standard (bupivacaine 1µg/ml). Each determination was taken as the mean of duplicate injections in most cases. The calibration curve was produced over the range 0.05-2µg/ml. Method validation studies were completed as described below.

HPLC system 2 used two Waters Model 501 pumps to deliver mobile phase to the analytical column. A Waters C18 Novapak Guard pak precolumn was used to protect the analytical column. The sample was injected using a Waters WISP 710B autosampler with a capacity for 48 samples. The sample size injected was 50µl. The detector was a Waters model 486 tunable absorbance detector and the eluent was monitored at 230nm. Instrument control, data acquisition, processing,

and custom reporting was handled using a NEC Powermate 386 computer and Waters Millennium 2010 (version 2.0) Chromatography Manager software (Waters, Marlborough, MA). In addition, this system also included Waters Millennium System Suitability software which provided for trend plotting, quality control, and method validation following GMP/GLP regulatory protocols. Therefore, in addition to the method validation procedures described below and performed with System 1, a GLP method validation program was developed for the analysis of cocaine and metabolites via HPLC. With this program, the chromatographic system was validated prior to unknown sample analysis to insure that samples were analyzed on a validated method. If the method failed the validation test, problems were corrected before analysis of unknowns.

Method Validation and Quality Control

Standard analytical procedures

Standard curves for quantitative analysis were constructed using bupivacaine as the internal standard for both HPLC analysis and for GC-MS. In each case the range of the standard curve, precision and accuracy, specificity and sensitivity were determined using the following standard procedures.

Extraction recovery

Extraction recovery of cocaine, benzoylecgonine, norcocaine, and cocaethylene was determined by comparison of the peak area observed for a nonextracted standard solution in an appropriate solvent injected directly onto the HPLC to the peak area observed with a solution prepared at the same

concentration in the appropriate biologic matrix and injected onto the HPLC following extraction. Extraction recovery was determined from all biological matrices used with five replicates at each concentration.

Range of standard curve and control samples

The standard curve was constructed so that the maximum calibrator was >20% of the expected maximum concentration and the minimum calibrator was at least 10% > than the minimum quantifiable value (10 x baseline noise). A 6-8 point calibration curve was prepared in the range 100-1750ng/ml for each compound (cocaine, benzoylecgonine, norcocaine, and cocaethylene) with a constant bupivacaine concentration. Control samples were included in the analysis of unknown samples by injection of a control for every 5-10 unknown samples injected. Stock solutions of 1mg/ml of cocaine, norcocaine, benzoylecgonine, and bupivacaine and 1.5mg/ml of cocaethylene were prepared in methanol. A mixed standard stock solution containing 10µg/ml of COC, BZE, NC, and CE was then prepared in methanol. Calibration standards in the range 100-1750ng/ml of COC, BZE, NC, and CE and 1000ng/ml of bupivacaine were prepared in methanol and evaporated to dryness. Standards were reconstituted in the HPLC mobile phase (for direct injection) or in the biological matrix (in preparation for solid-phase extraction).

Linearity and precision of standard curves

The standard curve was determined from the calibrators by a linear least squares fit to the equation $y = mx + b$, where x = concentration and y = ratio of drug

peak area to internal standard peak area. The regression line was not forced through zero. An "r" value between 0.97 to 1.0 was considered to represent acceptable linearity. Precision was assessed from the slope of replicate standard curves run on the same day and over a minimum two week period. The y-intercept must be less than 25% of the value of the minimum standard curve calibrator. Throughout the study period, changes in curve slope, y-intercept and residuals were assessed to check for occurrence of proportional or determinate error.

Accuracy and precision

Intraday and interday precision were determined on the standards. Replicate (n = 7) analysis of control samples was performed on the same day and over a minimum period of two weeks. Precision is expressed as the relative standard deviation of the intraday and interday replicate analysis for each control sample. Accuracy is expressed as the relative difference between the actual value and the mean measured intraday and interday value for each control. Minimal acceptable accuracy and precision criteria are defined based on the specific requirements of each study and for this study the minimal acceptable variation was 15% for interday replicate analysis.

Sensitivity

The limit of detection of each assay procedure was defined as three times the signal to noise ratio and the limit of quantitation as five times the signal to noise ratio determined by repetitive analyses of a drug free specimen. Sensitivity was documented by replicate analysis of blank (drug free) samples and samples

containing drug at minimal detectable and minimal quantifiable concentration in the appropriate biological matrix.

Specificity

Samples of the appropriate biological matrix known to be drug free were analyzed both with and without a known quantity of drug and/or metabolites added to the sample to demonstrate lack of interference from endogenous materials. Resolution of chromatographic peaks of drug, metabolites, internal standard and/or other eluting compounds was determined.

Gas Chromatography-Mass Spectrometry / HPLC Comparison Experiment

Instrumentation

A Finnigan MAT Incos 50 quadruple mass spectrometer (San Jose, CA), Hewlett-Packard Model 7673A autosampler and Hewlett-Packard Model 5890 gas chromatograph (Palo Alto, CA) were used for quantitative analysis of CO, BZE, NC, BUP, and CE. The compounds were separated on a 30 m X 0.32 mm i.d. fused silica capillary column coated with a 0.25µm film thickness of bonded-phase methyl silicone (DB-1) (J&W Scientific, Folsom, CA). The injector was operated in the splitless mode at 280°C, and helium was used as carrier gas at a column head pressure of approximately 6psi and flow rate of 1ml/sec. Specimens were injected at 50°C, the split valve opened after 1 minute, and the temperature was increased to 280°C at a rate of 33°C/min. The ion source was operated at 180°C, with an accelerating voltage of 70eV, 750µA filament current, and 1 kV electron multiplier voltage. Ion current was acquired at the following masses for cocaine and

benzoylecgonine: CO: 82, 182, 303 and BZE: 300, 316, 421. A multiple ion descriptor was determined for the other metabolites before other studies were done. The total scan time was 0.439 seconds.

Multiple ion descriptor

A multiple ion descriptor was determined for all compounds, including the internal standard. Using 100µg of drug diluted in 100µl of dimethylformamide (DMF), each sample was chromatographed and the full range was scanned. The multiple ion descriptor was selected based on the fragmentation pattern of the compounds. A minimum of three ions were chosen. Individual metabolites (except BZE) were also subjected to a derivitization procedure as described below to determine if the norcocaine, bupivacaine, or cocaethylene would derivitize.

Derivatization

Each sample for GC/MS was transferred to a silanized Reacti-vial™(Varian, Harbor City, CA) following SPE and the eluting buffer was evaporated at 60-70°C under a gentle nitrogen sweep. To this residue, 50µL of pentafluoropropionic anhydride (Aldrich, Milwaukee, WI) and 25µL of pentafluoropropanol (Aldrich) was added. The vial was tightly capped, vortex-mixed, and the derivatization reaction allowed to proceed for 20 minutes at 78°C, after which time the reagent was evaporated under a nitrogen sweep. The residue was reconstituted in 50µL of dimethylformamide prior to GC/MS analysis.

Experimental

A six point calibration curve was prepared in urine. A sample of drug-free urine was obtained from a volunteer. The urine was spiked with cocaine, benzoylecgonine, norcocaine, and cocaethylene to prepare a stock solution (10µg/ml). Bupivacaine (0.5µg/ml), the internal standard, was added to individual standards and samples prior to SPE. Concentrations were extracted in the range of 0-1500ng/ml. Samples were extracted via the solid-phase extraction method described above for urine. The SPE eluant was then evaporated to dryness under nitrogen, reconstituted in 100µl of methanol, and split into 2x50µl samples. These samples were then evaporated to dryness and one sample was reconstituted in 50µl HPLC mobile phase and subjected to HPLC analysis. The second sample was derivitized as described above, reconstituted in 50µl DMF, and subjected to GC/MS analysis.

A calibration curve for each compound was prepared on three separate days over a 2 week period using this method. Then, 10 urine samples identified as known cocaine positive were also extracted and analyzed by the procedure described for standards. Finally, interday variability was also determined by replicate analysis of 7 standard samples, split and analyzed according to the above procedure. The HPLC used for this experiment was System 2 described above. A statistical comparison of the GC/MS and HPLC data was performed for comparison of sensitivity, precision, quantitation, and reproducibility using paired T-tests and a standard error of the estimate determination.

Specific Aim #2

To characterize cocaine metabolism and distribution in the maternal-fetal unit using both human samples and animal models.

Human Studies -- Meconium and Urine

Two studies were done. In study 1, samples of infant urine and meconium were collected from 22 consecutive live births. Urine samples were screened using the Abbott AD_x^{TM} analyzer described above. Any positive samples were confirmed by HPLC, following solid-phase extraction, as described above under methods for specific aim #1. Meconium samples were analyzed by HPLC only (following solid-phase extraction (SPE)). In the second study, comprehensive meconium testing was compared to targeted urine immunoassay screening. For this study, all newborns delivered at a large, urban community hospital over a one month period and all newborns delivered at a large, suburban community hospital over a one month period were included. The total number of newborns studied was 312. Meconium was collected from all newborns and analyzed by HPLC following SPE. Newborn urine samples were collected only from those deliveries considered as high risk for gestational cocaine exposure. Risk factors identified were history of cocaine use, history of other illicit drug use, preterm labor, no prenatal care, unexplained hypertension, and abruptio placentae. Targeted urines were screened by immunoassay. The incidence of cocaine exposure was determined by each method and the test sensitivity was then calculated for each method based on the total number of newborns subsequently assigned to the "cocaine exposed" group.

Incidence and sensitivity were evaluated for statistically significant differences using chi-squared analysis.

Human Study -- Amniotic Fluid, Cord Blood, and Newborn's Urine

Samples of amniotic fluid, cord blood, and infant urine were obtained from 14 women who either reported a history of cocaine use at some time during pregnancy or who were strongly suspected of such use. These individuals were identified by the neonatologists. Samples were collected as described above at the beginning of the methods chapter. Cord blood (0.5-1ml) was analyzed by HPLC only (following SPE). Urine samples were screened using the Abbott AD_x™ analyzer and any positive samples were confirmed by HPLC (following SPE). All amniotic fluid samples were analyzed by HPLC (following SPE); however, these samples were also screened with the Abbott AD_x™ analyzer for method development purposes. All samples were analyzed for detection and quantification of cocaine and cocaine metabolites.

Specific Aim #3

To characterize cocaine metabolism and distribution with the co-ingestion of alcohol.

High Dose Cocaine--Mice

Two groups of male ICR mice (Harlan, Indianapolis,IN) with 18 mice per group were used. Group 1 mice were dosed with cocaine (55mg/kg IP). Group 2 mice were dosed with cocaine (55mg/kg IP) and pretreated with ethanol (3g/kg

gavage) 30 minutes prior to the cocaine dose. Mice were euthanized with CO_2 asphyxiation at time points of 15, 30, 60, 90, 120, and 180 minutes with 3 mice sacrificed at each time point. After sacrifice, blood was collected by cardiac puncture and the serum (300-500µl) was isolated. The brain was also harvested. Samples were stored frozen at -20°C until SPE and HPLC analysis and quantitation. Statistical analysis was performed using a one-way ANOVA to compare the mean drug concentration at each time point between groups 1 and 2.

Low Dose Cocaine--Mice

Two groups of male ICR mice (Harlan) with 18 mice per group were used. This experiment was designed as under "high dose" above except the cocaine dosage was lower and designed to more closely approximate human physiological attainable cocaine concentrations. Group 1 mice were dosed with cocaine (10mg/kg IP). Group 2 mice were dosed with cocaine (10mg/kg IP) and pretreated with ethanol (2g/kg gavage) 30 minutes prior to the cocaine dose. Mice were euthanized with CO_2 asphyxiation at time points of 15, 30, 60, 90, 120, and 180 minutes with 3 mice sacrificed at each time point. After sacrifice, blood was collected by cardiac puncture and the serum (300-500µl) was isolated. The brain was also harvested. In addition, serum ethanol concentrations were measured in a 10µl aliquot using a commercially available kit (Sigma, St. Louis, MO). Samples were stored frozen at -20°C until SPE and HPLC analysis and quantitation. Area-under-the curves for the time versus concentration plots were calculated using the trapezoidal rule and compared for statistically significant differences using a one-

way ANOVA. The time to peak concentration (Tmax) and the peak concentration (Cmax) were determined for each group from the plot of time versus concentration.

Pregnant Mice

Two groups of female ICR timed-pregnant mice (Harlan) were used with 15 mice per group. On day 17 of gestation, Group 1 mice were dosed with cocaine (5mg/kg tail vein IV). On day 17 of gestation, Group 2 mice were dosed with cocaine (5mg/kg tail vein IV) and pretreated with ethanol (2g/kg gavage) 30 minutes prior to the cocaine dose. Mice were euthanized with CO_2 asphyxiation at time points of 15, 30, 60, and 90 minutes with 4 mice sacrificed at each time point. After sacrifice, maternal blood was collected (300-500µl) by cardiac puncture and the maternal brain was harvested. The fetal brains were also harvested. All fetal tissues from the same dam were pooled and then split into 2 separate samples if sufficient tissue had been collected. Samples were stored frozen at -20°C until SPE and HPLC analysis and quantitation. Statistical analysis was performed between the groups using a one-way ANOVA to compare maternal and fetal brain cocaine concentrations and maternal blood cocaine and benzoylecgonine concentrations. In addition, elimination rates for cocaine were calculated on brain cocaine concentrations over time using a semi-log plot of time versus cocaine concentration. Elimination rates were evaluated by independent t-tests for statistically significant differences.

Benzoylecgonine Dosed Mice

To examine BZE transport across the blood brain barrier, male ICR mice (Harlan) were divided into 2 groups as described above for cocaine dosing. Group 1 mice were dosed with BZE (10mg/kg IP). Group 2 mice were dosed with BZE (10mg/kg IP) and pretreated with ethanol (2g/kg gavage) 30 minutes prior to the BZE dose. Mice were euthanized with CO_2 asphyxiation at time points of 15, 30, 60, 90, 120, and 180 minutes with 3 mice sacrificed at each time point. After sacrifice, blood was collected by cardiac puncture and the serum (300-500µl) was isolated. The brain was also harvested. Samples were stored frozen at -20°C until SPE and HPLC analysis and quantitation. Statistical analysis was performed using a one-way ANOVA to compare the mean drug concentration at each time point between groups 1 and 2.

Rat Acute and Chronic Dosing with Cocaine / Ethanol

Four groups of Sprague Dawley male rats were used for this experiment and with five rats per group. Groups 1 and 2 were "acute" groups. Group 1 received a single dose of cocaine (10mg/kg IP). Group 2 received a single dose of cocaine (10mg/kg IP) and of ethanol (1.5g/kg IP). Blood samples were collected at 15, 30, 60, 120, and 180 minutes. The brain was harvested after the last blood collection and the hypothalamus was sectioned from the rest of the brain. Samples were stored frozen at -20°C until extraction and analysis and quantitation by HPLC. Groups 3-4 rats were dosed daily for 24 days and were considered "chronic" groups. Group 3 was dosed with cocaine (10mg/kg IP) and saline (1.5g/kg IP).

Group 4 was dosed with cocaine (10mg/kg IP) and ethanol (1.5g/kg IP). Blood samples were collected at 15, 30, 60, 120, and 180 minutes. The brain was harvested after the last blood collection and the hypothalamus was sectioned from the rest of the brain. Samples were stored frozen at -20°C until extraction and analysis and quantitation by HPLC. Area-under-the curves for the time versus concentration plots were calculated and compared for statistically significant differences. In addition, the time to peak concentration (Tmax) and the peak concentration (Cmax) were determined for each group from the plot of time versus concentration.

Specific Aim #4

To examine the effect of cocaine, norcocaine, benzoylecgonine, and cocaethylene on T and B cell proliferation.

IM-9 B-Lymphoblastoid Human Cell Line

Cells

IM-9 cells (CCL#159, ATCC, Rockville,MD) were used to study the effect of cocaine and metabolites on B cell function. These cells have receptor sites for human growth hormone, IGFII, insulin, and calcitonin. The cells were cultured in suspension in a complete medium consisting of 90% RPMI1640 (supplemented with 100µg/ml streptomycin, 100 units/ml penicillin, and 2mmol L-glutamine) and 10% fetal bovine serum. The cell suspension was replenished every 48 hours.

Materials

Cocaine hydrochloride, benzoylecgonine, and phorbol 12-myristate 13-acetate (PMA) were obtained from Sigma (St. Louis, MO). Cocaethylene and norcocaine were obtained from the National Institute on Drug Abuse (NIDA, Rockville, MD). Drug solutions were sterile filtered through 0.2µm syringe acrodisc filters (Gelman Sciences, Ann Arbor, MI). RPMI 1640 medium (Gibco, Grand Island, NY) was supplemented with streptomycin (100µg/ml; Gibco), penicillin (100units/ml; Gibco), and L-glutamine (2mM; Gibco). IM-9 cells (CCL#159) were obtained from ATCC (Rockville, MD) and fetal bovine serum (FBS) from Hyclone Laboratories. [^{3}H]-thymidine (5.0 Ci/mmol) was obtained from Amersham Life Science, scintillation fluid from Research Products Division (Costa Mesa,CA), and cell culture plates were from Corning (Marietta, GA).

Drug Preparation

Cocaine hydrochloride, benzoylecgonine, and cocaethylene were dissolved in sterile phosphate buffered saline (PBS) at concentrations of 40X final well concentration. Norcocaine was dissolved in a PBS solution containing 1% methanol to aid solubility, then diluted to 40X final concentration in PBS. Then, all drug solutions were sterile filtered through 0.2µm syringe acrodisc filters. Solutions were then diluted just prior to use in complete RPMI serum-free medium (described under materials) to 4X final well concentration. Final well concentrations of drug solutions were in the range 0.01µg/ml - 10.0µg/ml with four concentrations of each

drug. Phorbol 12-myristate 13-acetate (40nM) was used as a negative control in proliferation studies.

[^{3}H] Thymidine Incorporation Assays

Proliferation assays were performed when the cells were in early log-phase growth. Cells were cultured in either RPMI 1640 serum-free medium or in RPMI 1640 medium supplemented with 2% fetal bovine serum (final well concentration) for 24 and 48 hours in a humidified, 37°C atmosphere with 5%CO_2 in air.

Cocaine, benzoylecgonine, norcocaine, and cocaethylene (50µl, 4X final well concentration) in serum-free RPMI 1640 medium were added in triplicate to the 96 well plate for final well concentrations of 0.01-10.0µg/ml. Drug-free controls and PMA controls were included. IM-9 cells were collected by centrifugation, washed once with serum-free medium, and resuspended at $2X10^5$ cells/ml (100µl added to each well). This resulted in $2X10^4$ cells per well. Viability and cell density were determined by trypan blue exclusion. Cells were cultured in 96 well plates in a final volume of 200µl. The cells were pulsed with 1µCi of [^{3}H]-thymidine for the last four hours of incubation. The cells were harvested onto glass fiber filters using a Brandel automatic cell harvester. The filters were dried overnight and then placed into scintillation vials with 4ml of scintillation fluid. The amount of incorporated tritiated thymidine (in counts per minute (CPM)) was determined for both the controls and the drug treated groups using standard liquid scintillation counting.

Preliminary experiments were completed to determine the best incubation conditions for experiments. Using the methods described, IM-9 cells were

incubated in varied conditions. Using, (1) Two cell types: Cells cultured prior to the experiments in RPMI 1640 serum-free medium or in complete medium; (2) Four medium types for experiments: In either RPMI 1640 serum-free medium or in RPMI 1640 medium supplemented with fetal bovine serum producing a final serum well concentration of 1, 2, or 5%; (3) Two time periods: 24 and 48 hours; (4) Three cell densities: $1X10^4$ cells/well, $2X10^4$ cells/well, or $5X10^4$ cells/well.

In addition, a series of experiments were conducted to find external controls to be included during the proliferation experiments with cocaine and metabolites. Compounds investigated as controls were tumor necrosis factor (TNFα), transforming growth factor (TGFβ_1), interleukin 6 (considered a B cell stimulating factor), dexamethasone, human growth hormone, and phorbol 12-myristate 13-acetate (PMA). Experimental conditions were: 4 concentrations of each compound, $2X10^4$ cells/well, 48 hour incubation with a 4 hour [^{3}H]-thymidine pulse, and in either 2%FBS RPMI 1640 medium or in RPMI 1640 serum-free medium. Cell proliferation responses were evaluated in order to choose a suitable control for cocaine and metabolite experiments.

Drug stability during the incubation period

Since cocaine and cocaethylene, and norcocaine are subject to hydrolysis to benzoylecgonine and norbenzoylecgonine respectively, drug stability for all compounds was monitored over the incubation period to insure that any effects observed were due to the specific drug studied and not a degradation product. Drug concentrations were also verified by HPLC prior to use. The HPLC system

used was System 1 described above. Stability of the drugs in medium, under incubation conditions, was determined by incubation of two concentrations of each drug in both serum-free and 2%FBS RPMI 1640 medium and both with or without cells for 48 hours. After 48 hours, the medium was removed from the wells with a pipet and stored frozen at -20°C pending HPLC analysis. Medium from wells containing cells was centrifuged (1000g, 10 minutes) and the supernatant removed for analysis by HPLC.

Statistics

The amount of incorporated tritiated thymidine (in CPM) was determined for both the controls and the drug treated groups. Treated cells were then expressed as the percentage of control for the respective treatment condition (incubation time and media type). Means and standard errors for each drug and each drug concentration were calculated using the percentage of control values from three separate experiments. One-way ANOVA was performed at each drug concentration.

Cord Blood PBMCs (Peripheral Blood Mononuclear Cells)

Sample collection

Cord blood (20ml) from both cocaine exposed (n=5) and non-exposed (n=5) newborns was collected in heparinized blood collection tubes immediately after delivery. Women who used cocaine during pregnancy were identified by chart review upon their arrival for labor and delivery. Cord blood mononuclear cells were isolated and stimulated with a mitogen to compare proliferative responses between

the cocaine exposed and non-exposed groups. Phytohemagglutinin was selected as the mitogen for these experiments because it stimulates the first signal transduction pathway in T cell activation.

Materials

Human albumin, histopaque 1077, phytohemagglutinin (PHA-L), RPMI 1640 medium, and [^{3}H]-thymidine were purchased from Sigma (St. Louis, MO). Scintillation fluid was from National Diagnostics (Atlanta, GA) and cell culture plates were from Corning (Marietta, GA).

PBMC culture and [^{3}H]-thymidine incorporation assays

Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll Hypaque density gradient centrifugation. Cord blood (up to 20ml) was diluted to 40ml with RPMI 1640 medium supplemented with 5% human albumin. Histopaque density gradient (10ml) was then layered under the blood. The sample was centrifuged for 30 minutes at 1200g to isolate the PBMCs. After centrifugation, the PBMCs form a layer between the histopaque density gradient and excess RPMI medium. This layer was removed with a sterile transfer pipet, diluted to 50ml with RPMI 1640 medium supplemented with 5% human albumin, and centrifuged for 10 minutes at 1200g. The cell pellet was resuspended at $1X10^6$ cells/ml (100µl pipeted to each well). This resulted in $1X10^5$ cells per well. Viability and cell density were determined by trypan blue exclusion. Cells were cultured in 96 well plates in a final volume of 200µl.

The PBMCs ($1x10^5$ cells/well) were then cultured with phytohemagglutinin (5µg/ml) and RPMI 1640 medium supplemented with 5% human albumin for 72 hours in a 37°C humidified atmosphere with 5% CO_2. PBMCs were also cultured without PHA to serve as unstimulated controls for the PHA stimulated cells. The cells were pulsed with 1µCi of [^{3}H]-thymidine for the last 24 hours of incubation. The cells were harvested onto glass fiber filters using a cell harvester. The filters were dried overnight and then placed into scintillation vials with 4ml of scintillation fluid. The amount of incorporated tritiated thymidine (in CPM) was determined for both the controls and the drug treated groups using standard liquid scintillation counting.

Statistics

The differences in cell proliferation, in response to stimulation by PHA, between the cocaine exposed versus non-exposed cord blood lymphocytes was evaluated using a one-way ANOVA.

CHAPTER 3
RESULTS

Specific Aim #1

Non-enzymatic Hydrolysis of Cocaine in Human Urine -- Analysis by AD_x™

Results (Tables 3-1, 3-2, 3-3) indicate cocaine spiked into human urine can be adequately hydrolyzed to benzoylecgonine for analysis by immunoassay. Ideal hydrolysis conditions were: hydrolysis pH 9.5 or greater, incubation time of at least 30 minutes at 50°C, and the sample adjusted to pH 8.0 in preparation for analysis by immunoassay. The next objective was to examine whether this procedure could be followed for analysis of actual clinical samples.

Table 3-1: Initial experimental conditions for hydrolysis.

Sample Description	Concentration reported by AD_x (ng/ml)
Medium Control	1440
Blank human urine	0
Cocaine spiked urine (1000ng/ml)	13
Cocaine spiked urine @ pH 8 and left at room temp. for 10min	82
Cocaine spiked urine @ pH 5.7 and left at room temp. for 10min	22
Cocaine and BZE spiked urine (5µg/ml)	High (>5000ng/ml)
BZE spiked urine (5µg/ml)	High (>5000ng/ml)

Table 3-2: Hydrolysis experiment 2 of cocaine to benzoylecgonine at varying pH after incubation for 60 minutes at 50°C.

Sample	Hydrolysis pH [a]	AD_x pH [b]	Concentration (ng/ml) [c]
Blank urine	----[d]	----	Low
Blank urine "hydrolyzed"	----	----	Low
COC spike - no hydrolysis	----	----	2
Medium Control	----	----	1417
Spike 1000ng/ml COC	9.5	7.1	1021
Spike 1000ng/ml COC	9.8	6.2	1071
Spike 1000ng/ml COC	10.2	6.4	932
Spike 1000ng/ml COC	10.2	7.9	962
Spike 1000ng/ml COC	10.5	8.1	765
Spike 1000ng/ml COC	10.5	6.6	679
BZE spike	----	----	High

[a] Hydrolysis pH is the pH to which the urine sample is adjusted with NaOH prior to hydrolysis incubation.

[b] AD_x pH is the pH to which the urine sample is adjusted with 6M HCl prior to analysis after the incubation period.

[c] Concentration reported is not quantitative due to sample dilution produced by pH adjustments. "Positive" results would indicate the sample would be submitted for confirmatory analysis and quantitation.

[d] ---- indicates not applicable to this sample.

Table 3-3: Final experiment to determine ideal cocaine hydrolysis conditions at 50°C.

Sample	Hydrolysis pH	Hydrolysis Time (min)	AD_x pH	Concentration (ng/ml)
Blank urine	unhydrolyzed	----	Unchanged	6
Blank urine	10.1	60	8.0	9
1000ng/ml	unhydrolyzed	----	Unchanged	65
100ng/ml	unhydrolyzed	----	Unchanged	5
Low control	unhydrolyzed	----	Unchanged	494
1000ng/ml	8.1	15	8.1	209
1000ng/ml	8.0	15	8.0	223
1000ng/ml	8.9	30	7.8	623
1000ng/ml	8.7	30	7.8	585
1000ng/ml	8.5	60	8.5	893
1000ng/ml	8.7	60	8.0	802
1000ng/ml	9.7	15	9.7	NA
1000ng/ml	9.2	30	9.2	843
1000ng/ml	9.8	15	8.3	623
1000ng/ml	9.4	30	7.9	909
1000ng/ml	10.1	15	7.4	574
1000ng/ml	10.4	30	10.4	862
100ng/ml	8.9	30	8.3	34
100ng/ml	8.5	60	8.5	69
100ng/ml	10.0	15	7.8	61
100ng/ml	9.7	30	7.0	96
100ng/ml	10.2	15	7.9	57
100ng/ml	10.2	30	8.3	93

Clinical Samples of Fetal Urine and Meconium

The presence of unmetabolized cocaine in newborn urine and meconium samples would limit the use of sensitive, rapid immunoassay screening. The results of experimental non-enzymatic hydrolysis of infant urine and meconium to improve detection of gestational cocaine exposure are shown in Table 3-4 and 3-5.

Table 3-4: BZE detection in meconium by immunoassay following hydrolysis.

Sample I.D.	BZE Concentration by the "direct" method (ng/g)	BZE concentration by the "hydrolysis" method (ng/g)
M0472	0	74
M0474	Low	Low
M0476	1	4
M0478	3	Low
M0479	287	281
Low control	485	----
cocaine spiked meconium (1µg)	89	952

Table 3-5: BZE detection in newborn urine by immunoassay following hydrolysis.

Sample I.D.	BZE Concentration by the "direct" method (ng/g)	BZE Concentration by the "hydrolysis" method (ng/g)
U0439	185	34
U0440	0	4
U0443	2	3
U0446	2	10
U0448	4935	1830
U0449	1	5
U0450	6	1
U0451	6	5
U0455	22	169
U0456	158	59
High Control	2832	----

Results of the hydrolysis experiments found that this method did not improve detection of gestational cocaine exposure in actual clinical samples. The method followed to complete the hydrolysis experiments was as labor-intensive as the solid-phase extraction and HPLC analysis described in Chapter 2, yet did not improve detection. For these reasons, this method was discontinued.

Solid-Phase Extraction Procedures for Biological Samples

Solid-phase extraction was the analytical method used to extract cocaine and metabolites from the biological matrices. Methods used for SPE are as written in Chapter 2. The percentage of recovery of drug from each biological matrix is

summarized in Tables 3-6 and 3-7 . Extraction recovery was satisfactory with all methods. Extraction recovery from placenta and liver tissue was variable and requires the use of an internal standard.

Table 3-6: Extraction recovery of cocaine and metabolites (1µg) from biological matrices.

Matrix	BZE[a]	COC[a]	NC[a]	CE[a]	BUP[a]
Meconium	81±11	87±9	88±7	91±7	91±5
Urine	84±9	92±4	86±4	93±6	89±4
Amniotic Fluid	62±12	86±8	----[b]	----	88±5
Whole Blood	75±8	86±4	90±3	91±8	85±4
Cord Blood	80±11	88±8	----	----	85±6
Serum	83±9	93±6	92±7	96±6	93±3

[a] Recovery is reported as % recovery ± % std. dev. of recovery from n=5 determinations.

[b] ---- indicates recovery not determined for this compound.

Table 3-7: Extraction recovery of cocaine and metabolites (1µg) from brain tissue.

Extraction Method	BZE[a]	COC[a]	NC[a]	CE[a]	BUP[a]
1--Digested	85±8	89±6	----[b]	----	90±4
2--Undigested	81±6	91±7	88±6	90±5	85±5

[a] Recovery is reported as % recovery ± % std. dev. of recovery from n=5 determinations for method 1 and n=3 determinations for method 2.

[b] ---- indicates recovery not determined for this compound.

Results of extraction of cocaine and metabolites from brain tissue indicated that digestion was not required. Therefore, solid-phase extraction method 2 was utilized for all brain extractions for this project. In brief, brain tissue was homogenized with phosphate buffer (3ml) and internal standard (10µl), centrifuged for 10 minutes, and the supernatant extracted using SCX columns. However, fetal brain tissue was sufficiently fluid such that the uncentrifuged homogenate could be extracted directly without blocking the SCX column. This method was followed for extraction of fetal brain tissues under Specific Aim#3.

High Performance Liquid Chromatography (HPLC)

The HPLC mobile phase providing the best resolution of drugs and endogenous matrix interferences was Mobile phase 4 which consisted of 0.025M potassium phosphate buffer : acetonitrile : butylamine (81:18:1) with the final pH adjusted to 3.0 with concentrated orthophosphoric acid. This mobile phase provided excellent chromatographic performance with both analytical columns used throughout the project. A typical HPLC chromatogram from HPLC system 1 is shown in Figure 3-1. In addition, system 1 employs a multiwavelength detector which allows for acquiring full spectra from 190-400nm (Figure 3-2) and the generation of the ultra-violet (UV) and derivative spectrum for each peak in the chromatogram (Figure 3-3 and Figure 3-4).

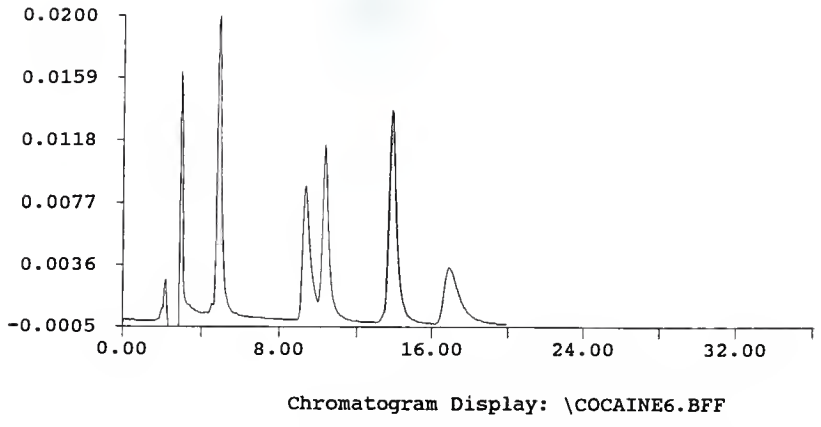

Figure 3-1: HPLC chromatogram of cocaine and metabolites at 230nm (system 1).

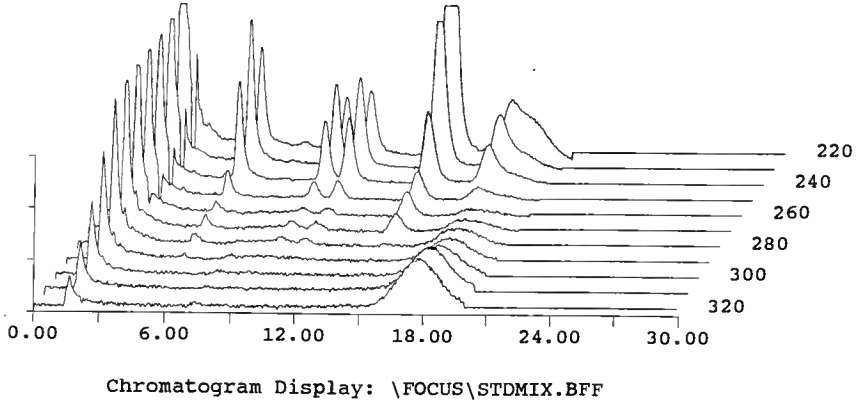

Figure 3-2: Full spectrum of standard solution of cocaine and metabolites.

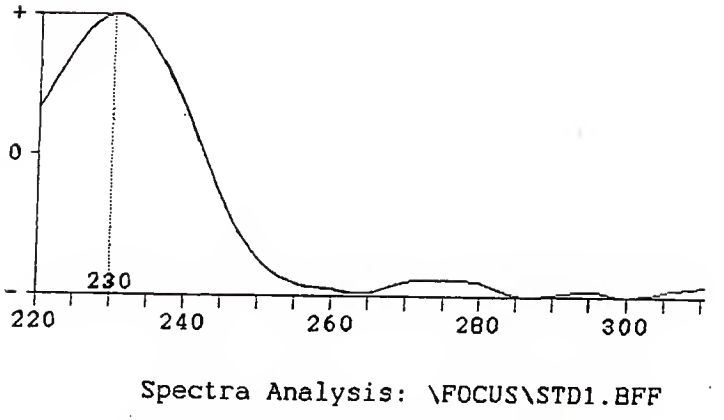

Figure 3-3: UV spectrum of a standard solution of cocaine.

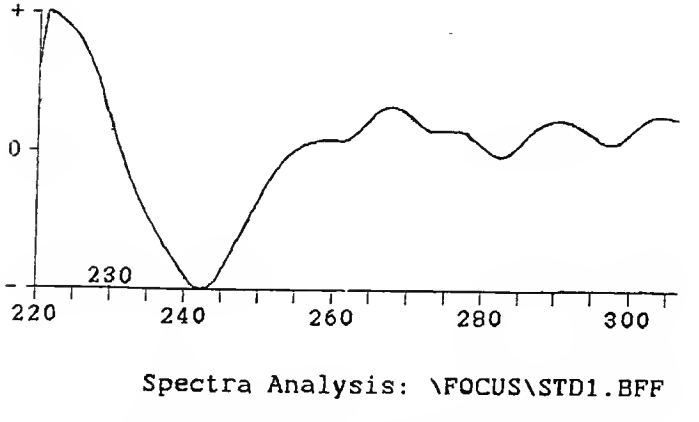

Figure 3-4: UV derivative spectrum of a standard solution of cocaine.

HPLC system 2 employs a tunable absorbance detector which is operated at 230nm for detection of cocaine and metabolites. A typical HPLC chromatogram is shown in Figure 3-5. System 2 also employs computer software to manage data acquisition and processing as well as quality control monitoring to evaluate chromatographic performance prior to and during sample analysis. Method validation information acquired after analysis of a set of standard samples for method calibration of cocaine and metabolites is shown in Figure 3-6 and Figure 3-7. This data was evaluated after the calibration to insure that the variations in column efficiency, resolution, and retention times were less than 10% and therefore indicating suitable chromatographic performance for the analysis of unknown samples.

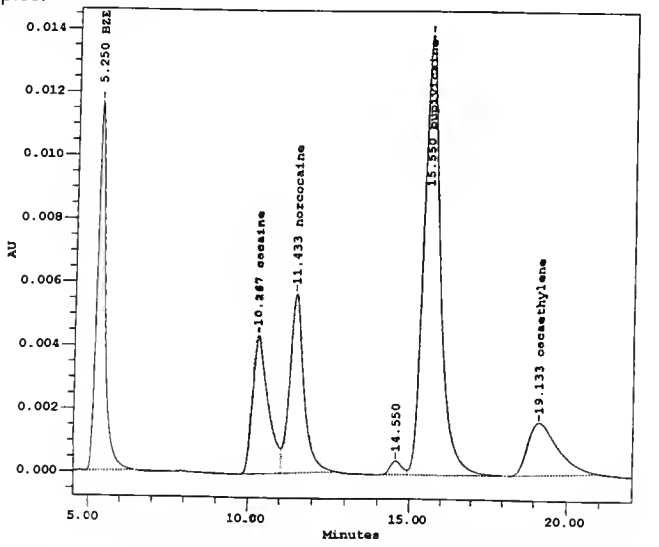

Figure 3-5: HPLC chromatogram of cocaine and metabolites (system 2).

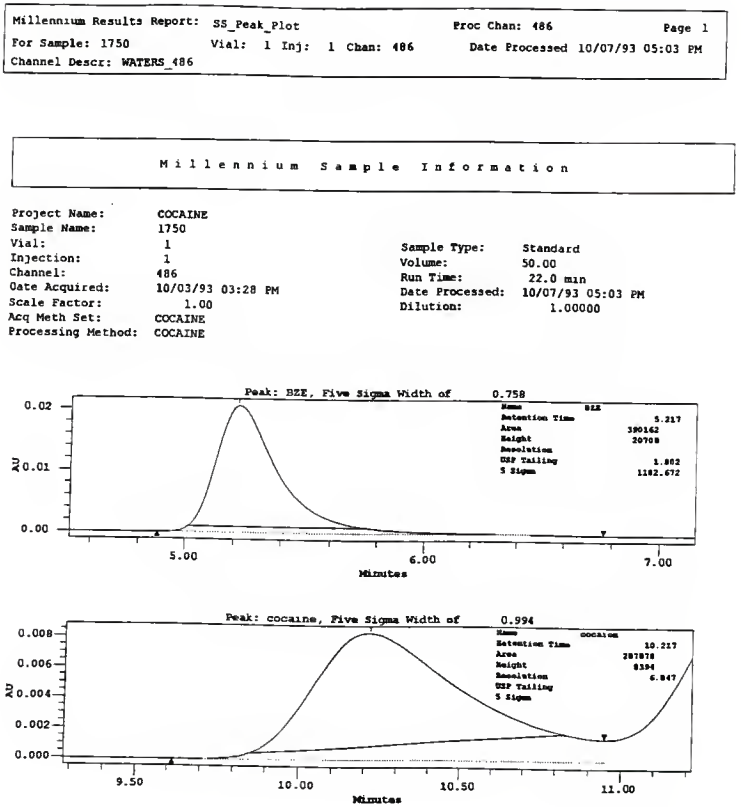

Figure 3-6: Validation report to monitor the chromatographic system.

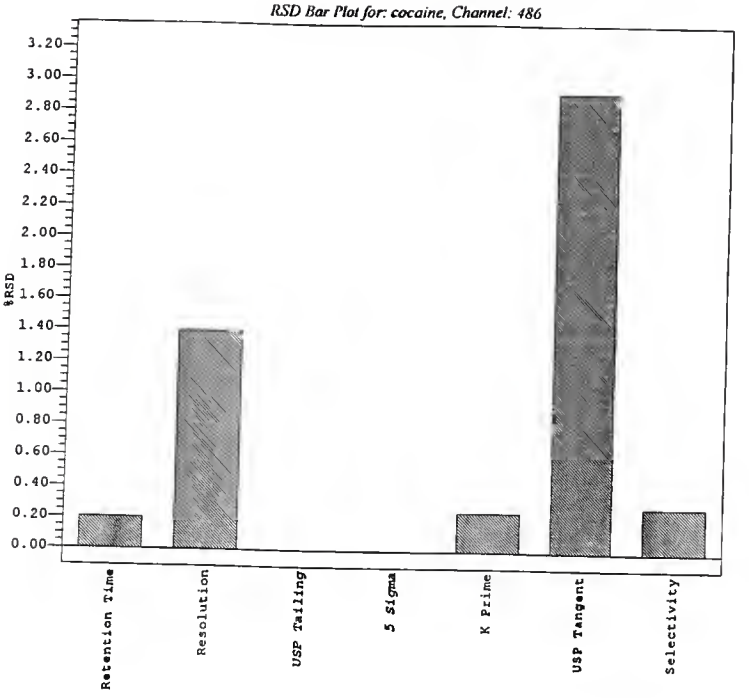

Figure 3-7: Method validation bar graph indicating with-in run variability.

The linearity and precision of standard curves of cocaine, benzoylecgonine, norcocaine, and cocaethylene with bupivacaine as an internal standard were routinely determined throughout the course of the project. A representative calibration curve is shown in Figure 3-8 for cocaine with the data shown in Table 3-8. Representative calibration curves for the metabolites are in Appendix A.

Table 3-8: Representative standard calibration data for cocaine.

Concentration (ng/g)	Average Response	Standard Deviation	n
100	320	10	2
250	650	10	2
500	1440	10	2
750	1770	20	2
1000	2400	10	2
1250	2910	0	2
1500	3490	20	2
1750	4030	10	2

The data is fit to the equation y=b + mx where m=slope and b=intercept.

Results of this calibration show: slope = 2.223
Intercept =68

The correlation coefficient (r) = 0.9984

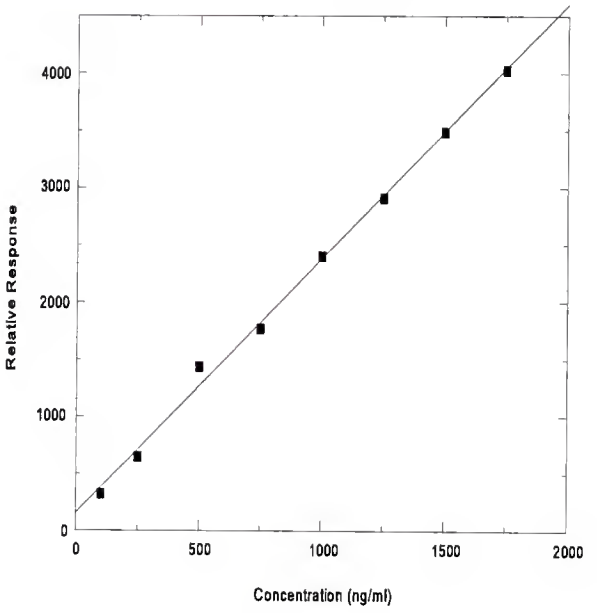

Figure 3-8: Representative calibration curve for cocaine.

Throughout the study period, changes in curve slope, y-intercept, and correlation coefficient were assessed (Table 3-9). Results indicate that the average deviation of slope for all compounds was less than 25% over a two-year period. These values were also determined utilizing two different HPLC systems, minor alterations in mobile phase, and two different types of analytical columns.

Table 3-9: Variation in slope, y-intercept, and correlation coefficient over time.

		BENZOYLECGONINE			COCAINE		
Date	HPLC System	slope	y-int[a]	r	slope	y-int[a]	r
3/30/92	1	3.71	-25	0.9825	2.75	15	0.9891
5/26/93	1	----[b]	----	----	3.88	40	0.9927
6/3/93	1	4.60	-24	0.9901	2.40	-70	0.9954
6/10/93	1	4.77	-31	0.9960	2.99	-16	0.9810
6/24/93	1	5.03	-33	0.9962	3.04	-26	0.9897
7/20/93	1	3.99	10	0.9976	2.01	7	0.9995
7/22/93	1	4.21	114	0.9669	2.83	-1	0.9996
12/1/93	1	4.73	75	0.9830	3.28	82	0.9952
7/13/93	2	3.36	1	0.9991	2.65	-33	0.9996
8/13/93	2	3.56	-2	0.9977	2.68	11	0.9930
10/4/93	2	3.92	33	0.9990	2.90	33	0.9989
2/21/94	2	3.89	48	0.9989	2.23	68	0.9984
5/20/94	2	4.80	-52	0.9896	3.80	-39	0.9974
8/10/94	2	4.82	-22	0.9998	3.51	9	0.9993
12/21/94	2	4.80	-17	0.9993	3.54	2	0.9993
10/26/94	2	5.61	-76	0.9923	4.41	-76	0.9944

[a] y-intercept values are reported in units of concentration.

[b] ---- indicates determination not made on this date.

Table 3-9--Continued.

		NORCOCAINE			COCAETHYLENE		
Date	HPLC System	slope	y-int[a]	r	slope	y-int[a]	r
3/30/92	1	----[b]	----	----	----	----	----
5/26/93	1	----	----	----	----	----	----
6/3/93	1	1.90	-13	0.9959	3.20	-138	0.9988
6/10/93	1	4.22	7	0.9840	3.14	-143	0.9764
6/24/93	1	4.66	-67	0.9990	3.23	-156	0.9997
7/20/93	1	1.73	49	0.9957	2.49	-118	0.9759
7/20/93	1	3.70	-15	0.9990	2.93	-143	0.9913
12/1/93	1	3.42	111	0.9962	2.69	58	0.9980
7/13/93	2	2.93	-24	0.9990	2.38	-33	0.9997
8/13/93	2	2.79	20	0.9951	1.71	-25	0.9962
10/4/93	2	3.20	18	0.9990	2.23	-53	0.9988
2/21/94	2	3.14	20	0.9990	2.01	49	0.9977
5/20/94	2	4.03	-47	0.9972	2.90	-100	0.9959
8/10/94	2	4.36	-21	0.9998	2.82	-113	0.9984
12/21/94	2	4.33	-14	0.9996	2.76	-93	0.9982
10/26/94	2	4.37	-101	0.9919	2.69	-93	0.9944

[a] y-intercept values are reported in units of concentration.

[b] ---- indicates determination not made on this date.

Intraday and interday quantiation variability were also assessed throughout the course of the study for both HPLC systems to determine the precision of the method (Tables 3-10, 3-11, 3-12). Raw data points are tabulated in Appendix B.

Table 3-10: Interday variability for HPLC system 1 at 1000ng/ml.

	Benzoylecgonine	Cocaine	Norcocaine	Cocaethylene
mean	1033	1044	1037	1005
Std.Dev.	106	94	114	140
%RSD	10.3	9.0	11.0	13.9
n	9	10	8	8

Table 3-11: Interday variability for HPLC system 2 at 1000ng/ml.

	Benzoylecgonine	Cocaine	Norcocaine	Cocaethylene
mean	1012	968	1065	991
Std.Dev.	29	34	89	115
%RSD	2.9	3.5	8.4	11.6
n	13	13	13	13

Table 3-12: Intraday variability for HPLC system 2 at 1000ng/ml.

	Benzoylecgonine	Cocaine	Norcocaine	Cocaethylene
mean	993	968	1050	976
Std.Dev.	11	24	89	27
%RSD	1.1	2.7	3.1	2.8
n	11	11	11	11

Method sensitivity and specificity were determined to demonstrate the limit of detection and the lack of interference with endogenous materials in the biological matrix. The HPLC methods provided adequate specificity for all compounds of interest; however, GC/MS demonstrated the best specificity of all analytical methods used.

Table 3-13: Limits of detection (LOD) and limits of quantitation (LOQ).

	LIMITS (ng/ml)							
Analytical Method	BZE		Cocaine		Norcocaine		CE	
	LOD	LOQ	LOD	LOQ	LOD	LOQ	LOD	LOQ
HPLC System 1	1	5	1	5	1	5	4	20
HPLC System 2	1	5	1	5	1	5	2	10
GC/MS	1	5	2	10	10	50	1	5

Gas Chromatography-Mass Spectrometry / HPLC Comparison

Results of the experiment to compare HPLC analysis of cocaine and metabolites to the "gold standard" GC/MS indicate that HPLC is a valid method of analysis. However, in situations where structural identity is required for confirmation purposes (for example, in the analysis of unknown urine samples for drugs of abuse), GC/MS is the preferred method of analysis. In the research laboratory, HPLC using photodiode array or multiwavelength detection can provide limited specificity via UV and derivative spectra and can be used for analysis of samples

from designed experiments (for example, when mice are injected with a known quantity of cocaine). The multiple ion descriptors for mass spectral analysis are illustrated in Table 3-14. Each compound, except the internal standard, was described by three major ions each of which must be present for a positive identification. Calibration results are compiled in Table 3-15 and include mean deteminations of slope, y-intercept, and correlation coefficient for the three separate calibrations by each method. Sensitivity of the analytical methods was compared by paired T-tests on the mean slope determinations (Table 3-16). Precision was evaluated by a paired T-test on the interday variabilities of the methods (Table 3-17). Quantitation of benzoylecgonine from human urine samples, previously identified as positive by a certified drug testing laboratory, showed excellent correlation between the methods (Figure 3-9). Results indicate that GC/MS demonstrated better precision than HPLC, but the methods provide equivalent sensitivity.

Table 3-14: Multiple ion descriptors for GC/MS analysis.

Compound	m/z monitored		
Benzoylecgonine - derivitized	300[a]	316	421
Cocaine	182[a]	198	303
Cocaethylene	196[a]	272	317
Bupivacaine	140[a]		
Norcocaine - derivitized	194[a]	313	435
Norcocaine	136[a]	168	289

[a] Mass used for regression analysis

Table 3-15: Calibration curve comparisons for GC/MS and HPLC (n=3 calibrations).

	Compound	Slope		Intercept (ng/ml)		r	
		Mean	SD	Mean	SD	Mean	SD
HPLC	BZE	6.37	0.22	-12	15	0.9972	0.0019
	Cocaine	3.90	0.15	4	18	0.9972	0.0034
	NOR	5.31	0.21	-41	8	0.9970	0.0074
	CE	9.86	0.295	3	4	0.9972	0.0035
GC/MS							
	D-BZE	6.63	1.30	27	70	0.9954	0.0111
	Cocaine	6.07	0.85	25	62	0.9896	0.0168
	D-NOR	1.48	1.59	-34	86	0.9980	0.1009
	CE	8.37	0.49	51	76	0.9942	0.0068

Table 3-16: Comparison of method precision.

Analytical Method	Inter-day Variability (%RSD)			
	Benzoylecgonine	Cocaine	Norcocaine	Cocaethylene
HPLC	4.98 **	6.99 **	3.79 **	7.18 **
GC/MS	1.98	8.28	3.00	5.99

** $p < 0.05$ using Paired T-test

Table 3-17: Comparison of method sensitivity.

Benzoylecgonine	Cocaine	Norcocaine	Cocaethylene
Equivalent	GC/MS **	HPLC **	Equivalent

** $p < 0.05$ using Paired T-test

The standard error of the estimate (Table 3-18) was calculated at the 95% confidence interval for each drug. Three calibrations were done using each method (HPLC and GC/MS) for each drug. The slopes and intercepts were determined by linear regression (y=mx+b). Then, using the calculated values of slope and intercept, the "calculated" concentration was determined. The "calculated" values were than plotted on the y-axis and the "actual" concentrations (50-1500ng/ml) were plotted on the x-axis. A linear regression of these plotted data yielded the calculation of the standard error of the estimate.

Table 3-18: Standard error of the estimate (ng/ml) for the calculated versus actual calibration concentrations (residuals) by GC/MS and HPLC. Calculated concentations determined by solving the y=mx+b equation following method calibration.

Method and Calibration #	Cocaine	BZE	Norcocaine	Cocaethylene
GC/MS #1	142.46	7.56	----[a]	43.57
HPLC # 1	62.48	62.91	38.05	33.38
GC/MS #2	50.24	106.78	----	86.42
HPLC #2	62.05	47.37	17.31	51.98
GC/MS #3	97.16	16.88	57.30	91.78
HPLC #3	28.88	48.05	85.81	68.58

[a] ---- indicates value not calculated.

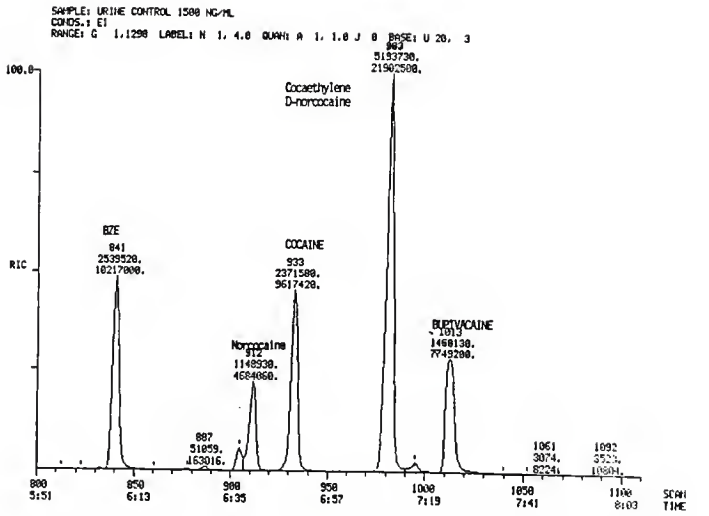

Figure 3-9: Gas chromatogram of cocaine and metabolites after derivitization.

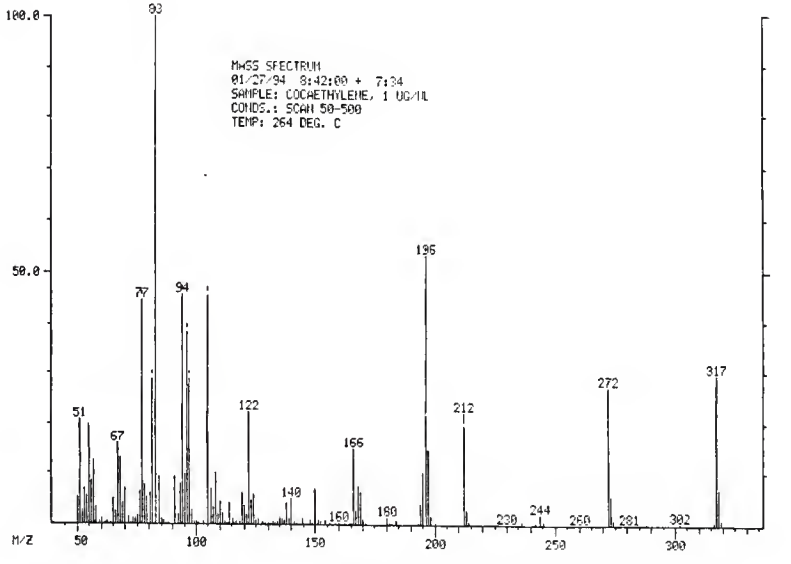

Figure 3-10: Mass spectrum of cocaethylene extracted from human urine.

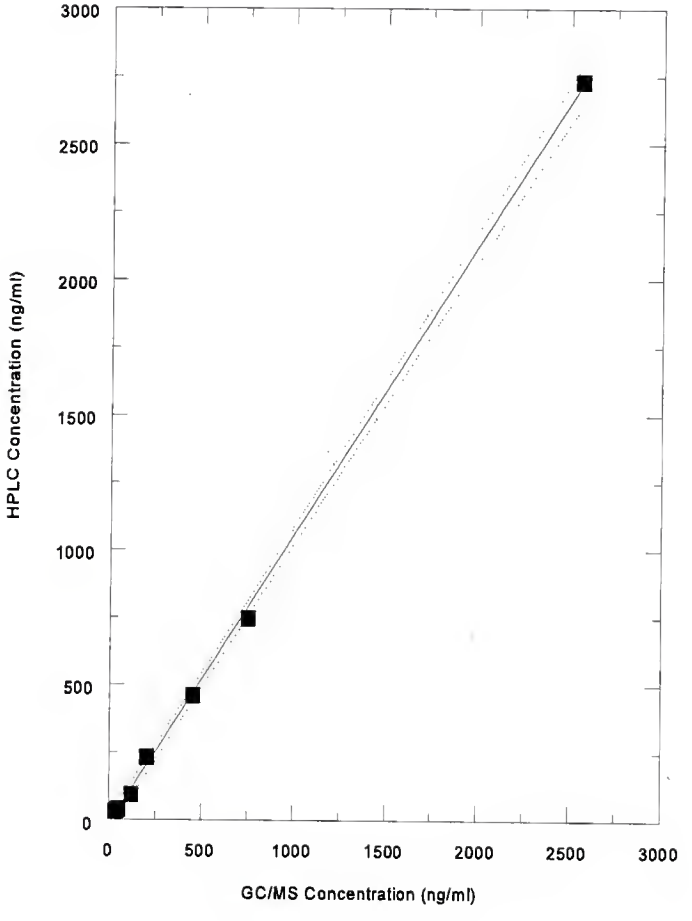

Figure 3-11: Method comparison for BZE quantitation in urine from human cocaine users.

Specific Aim #2

Human Studies -- Meconium and Urine

Results of analyses of newborn urine and meconium are in agreement with previously published studies indicating meconium is a better matrix for detection of prenatal cocaine exposure (Tables 3-19). In addition, the procedure of "targeting" women or their newborns for urine toxicology testing was evaluated against comprehensive meconium testing for identifying the incidence of cocaine exposed newborns (Table 3-20).

Table 3-19: Analysis of 22 paired samples of newborn meconium and urine.

Both Negative	Both Positive	-ve urine/+ve meconium	+ve urine/-ve meconium
18	2	2	0
Total positive = 4/22 = 18.2%			

Of 312 infants tested by comprehensive meconium testing or targeted urine immunoassay screening, 62 were identified as cocaine exposed by either maternal report of drug use or detection by either method of analysis. When the cocaine exposed group was evaluated for presence of clinical risk factors, it was found that 69% presented no risk factor. Of the 53 positive meconium samples, cocaine was detected in 40 (75%), benzoylecgonine in 36 (68%), norcocaine in 12 (23%), and cocaethylene in 1 (2%). Cocaine concentrations ranged from 140-3498ng/g, BZE from 92-8433ng/g, and norcocaine from trace-1357ng/g.

Table 3-20: Targeted urine screening versus comprehensive meconium testing.

	Targeted Urine (EMIT)	Comprehensive Meconium (HPLC)	p[a]
Incidence	10/312 (3.2%)	53/312 (17.0%)	0.0001
Sensitivity	10/62 (16.1%)	53/62 (85.5%)	0.0001
	Total cocaine exposed (by both methods)= 62/312 = 19.9%		

[a] p values were determined by chi-squared analysis.

Human Studies -- Amniotic Fluid and Cord Blood

Analyses of newborn urine, cord blood, and amniotic fluid from cocaine exposed newborns and their mothers indicate the highest concentrations of benzoylecgonine (the major cocaine metabolite) are found in amniotic fluid. The fetus is continually exposed to amniotic fluid throughout gestation. Results also used in evaluation of hypothesis (1) are reported under results for specific aim #3.

Table 3-21: Human study using biological fluids from 14 women with a history of cocaine use during pregnancy

Fluid Type	# Confirmed cocaine exposed	BZE Concentration Range (ng/ml)
Amniotic Fluid	10/14[a]	420 - 3100 ng/ml
Cord Blood	6/8[a]	120 - 770 ng/ml
Infant urine	5/12[a]	100 - 1110 ng/ml
4/14 individuals had all fluids confirmed cocaine unexposed.		
3/12 individuals had a negative newborn urine but a positive amniotic fluid.		
Unmetabolized cocaine was detected in 4/14 amniotic fluid samples.		

[a] Refers to the number exposed/the number of samples available for testing.

Specific Aim #3

A series of experiments were completed to evaluate the potential effects of the cocaine-ethanol interaction. An initial experiment with mice using near-lethal doses of cocaine determined that ethanol had a marked effect on cocaine metabolism resulting in increased concentrations of cocaine in both serum and brain. Next, a second experiment was completed with mice to monitor this interaction at cocaine doses designed to approximate human physiological cocaine concentrations. In this experiment, ethanol pretreatment increased peak serum cocaine concentrations approximately 5-fold and increased peak brain cocaine concentrations approximately 6-fold. In the rat, ethanol pretreatment had a similar effect, increasing blood cocaine concentrations.

High Dose Cocaine in Mice

Male mice were injected with sub-lethal doses of cocaine with or without a concurrent ethanol dose and sacrificed at time-points up to 180 minutes. Serum and brain samples were analyzed for cocaine, benzoylecgonine, norcocaine, and cocaethylene concentrations (Tables 3-22 to 3-27).

Table 3-22: Drug concentrations in SERUM (ng/ml) following an acute cocaine dose (55mg/kg IP).

Sample	BZE	Cocaine	Norcocaine	Cocaethylene
1-15	2620	1710	560	ND[a]
1-30	5690	1248	560	ND
1-60	----[b]	----	----	----
1-90	992	ND	ND	ND
1-120	450	ND	ND	ND
1-180	609	ND	ND	ND
2-15	1203	928	375	ND
2-30	1548	368	85	ND
2-60	1063	296	ND	ND
2-90	1722	43	21	ND
2-120	----	----	----	----
2-180	----	----	----	----
3-15	4265	622	188	ND
3-30	1419	142	70	ND
3-60	----	----	----	----
3-90	556	ND	ND	ND
3-120	1386	ND	ND	ND
3-180	140	ND	ND	ND

[a] ND indicates compound not detected.

[b] ---- indicates sample not available for measurement at this time point.

Table 3-23: Drug concentrations in SERUM (ng/ml) following an acute dose of cocaine (55mg/kg IP) and of ethanol (3g/kg gavage).

Sample	BZE	Cocaine	Norcocaine	Cocaethylene
1-15	862	3935	586	194
1-30	1054	526	185	128
1-60	2080	302	65	ND[a]
1-90	1777	77	25	ND
1-120	114	ND	ND	ND
1-180	----	----	----	----
2-15	925	3586	497	247
2-30	1009	752	330	106
2-60	1071	59	98	ND
2-90	1743	40	66	ND
2-120	911	ND	ND	ND
2-180	----	----	----	----
3-15	1172	522	188	30
3-30	708	238	68	43
3-60	----	----	----	----
3-90	1230	22	Trace	ND
3-120	----	----	----	----
3-180	----	----	----	----

[a] ND indicates compound not detected.

[b] ---- indicates sample not available for measurement at this time point.

Table 3-24: Drug concentrations in BRAIN (ng/g) following an acute dose of cocaine (55mg/kg IP).

Sample	BZE	Cocaine	Norcocaine	Cocaethylene
1-15	1224	14150	3683	ND[a]
1-30	1399	16348	5066	ND
1-60	481	2216	480	ND
1-90	610	869	320	ND
1-120	665	356	201	ND
1-180	680	282	152	ND
2-15	1771	21874	5834	ND
2-30	395	5698	1036	ND
2-60	617	684	404	ND
2-90	489	1165	229	ND
2-120	462	501	127	ND
2-180	490	178	98	ND
3-15	539	8040	1771	ND
3-30	821	8946	1570	ND
3-60	424	4016	539	ND
3-90	557	881	354	ND
3-120	525	463	261	ND
3-180	397	191	128	ND

[a] ND indicates compound not detected.

[b] ---- indicates sample not available for measurement at this time point.

Table 3-25: Drug concentrations in BRAIN (ng/g) following an acute dose of cocaine (55mg/kg IP) and of ethanol (3g/kg gavage).

Sample	BZE	Cocaine	Norcocaine	Cocaethylene
1-15	869	13656	3346	738
1-30	745	7522	1823	626
1-60	1055	1388	726	394
1-90	594	1534	477	295
1-120	303	208	194	ND[a]
1-180	340	128	72	ND
2-15	1253	37808	3564	1224
2-30	1328	16456	4188	1163
2-60	944	2534	921	344
2-90	914	991	401	256
2-120	414	346	176	ND
2-180	433	64	48	ND
3-15	1510	21238	4332	295
3-30	802	7200	1719	540
3-60	762	3442	804	522
3-90	514	908	298	ND
3-120	190	426	116	ND
3-180	550	146	83	ND

[a] ND indicates compound not detected.

Table 3-26: Mean drug concentrations in SERUM (ng/ml) following an acute dose of cocaine (55mg/kg IP) with or without ethanol (3g/kg gavage).

	Time	BZE		Cocaine		Norcocaine		Cocaethylene	
	(min)	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Cocaine only	15	2696	885	1087	324	374	107	ND[a]	
	30	2886	1403	586	337	238	161	ND	
	60	1063	0	296	0	102	0	ND	
	60	1090	340	43	0	21	0	ND	
	120	918	382	ND		ND		ND	
	180	375	191	ND		ND		ND	
Cocaine and Ethanol									
	15	986	95	2681	1084	424	121	157	65
	30	924	109	505	109	194	76	92	25
	60	1576	412	181	95	82	13	ND	
	90	1583	177	46	16	31	19	ND	
	120	513	325	ND		ND		ND	
	180	----[b]		----		----		----	

[a] ND indicates compound not detected.

[b] ---- indicates sample not available for measurement at this time point.

Table 3-27: Mean drug concentrations in BRAIN (ng/g) following an acute dose of cocaine (55mg/kg IP) with or without ethanol (3g/kg gavage).

	Time	BZE		Cocaine		Norcocaine		Cocaethylene	
	(min)	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Cocaine only	15	1178	356	14688	4003	3763	1174	ND[a]	
	30	872	291	10331	3151	2557	1264	ND	
	60	507	57	2305	963	474	39	ND	
	90	552	35	972	57	301	37	ND	
	120	551	42	440	75	196	67	ND	
	180	522	114	217	37	126	16	ND	
Cocaine and Ethanol									
	15	1211	186	24234	7131	3747	299	752	268
	30	958	186	10393	3033	2577	806	776	195
	60	920	85	2455	594	817	57	420	53
	90	674	122	1144	196	392	52	184	93
	120	315	66	327	64	162	24	ND	
	180	429	71	113	25	68	10	ND	

[a] ND indicates compound not detected.

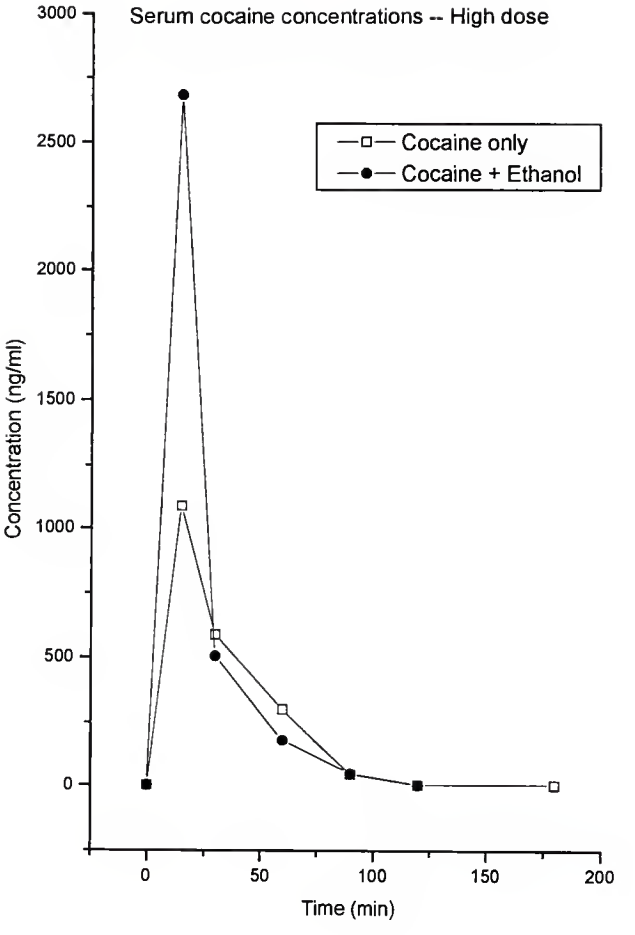

Figure 3-12: Serum cocaine concentrations in mice following a high dose of cocaine (55mg/kg) with or without an ethanol dose.

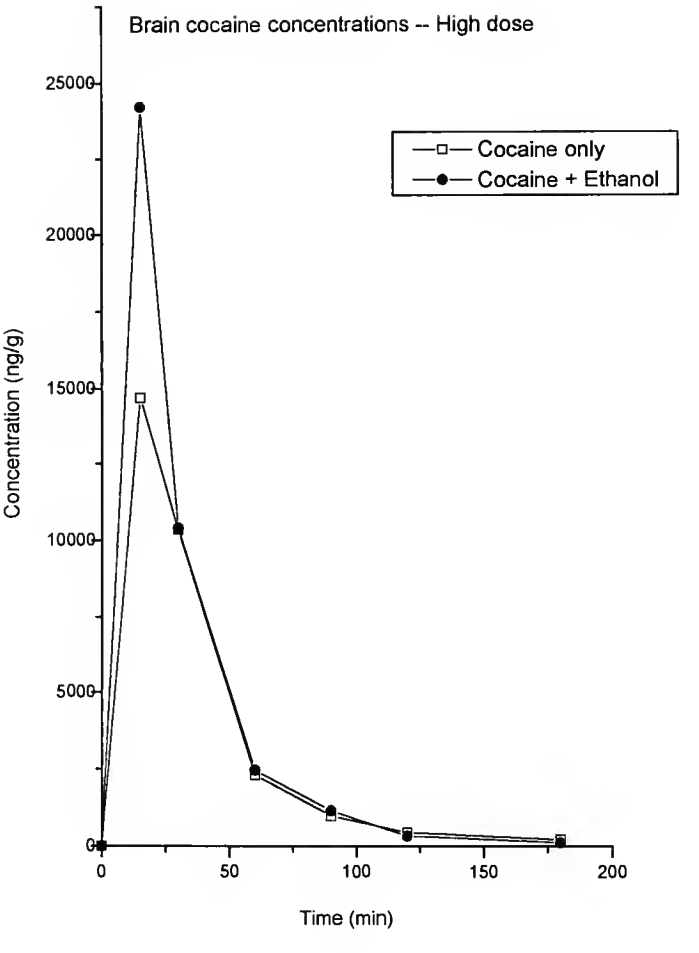

Figure 3-13: Brain cocaine concentrations in mice following a high dose of cocaine (55mg/kg) with or without an ethanol dose.

Low Dose Cocaine in Mice

Male mice were injected with low doses of cocaine with or without a concurrent ethanol dose and sacrificed at time-points up to 180 minutes. Serum and brain samples were analyzed for cocaine, benzoylecgonine, norcocaine, and cocaethylene concentrations (Tables 3-28 to 3-31). Norcocaine and cocaethylene were not detected in the mice injected with these lower doses.

Table 3-28: Mean cocaine concentrations in BRAIN (ng/g) following an acute dose of cocaine (10mg/kg IP) with or without ethanol (2g/kg gavage).

	Time	Raw Data			Cocaine (ng/g)	
	(min)	M1	M2	M3	Mean	SE
Cocaine only	15	10	6	70	29	21
	30	327	314	187	276	45
	60	58	65	42	55	7
	90	42	44	25	37	6
	120	ND[a]	ND	ND	ND	
	180	ND	ND	ND	ND	
Cocaine and Ethanol						
	15	1205	2915	1514	1878	526
	30	178	231	154	188	22.7
	60	20	32	26	26	3.5
	90	10	17	12	13	2.1
	120	5	10	8	7.7	1.4
	180	ND	ND	ND	ND	

[a] ND indicates compound not detected.

Table 3-29: Mean BZE concentrations in BRAIN (ng/g) following an acute dose of cocaine (10mg/kg IP) with or without ethanol (2g/kg gavage).

	Time	Raw Data			BZE (ng/g)	
	(min)	M1	M2	M3	Mean	SE
Cocaine only	15	99	76	46	74	15.3
	30	82	75	49	69	10.0
	60	44	43	72	53	8.6
	90	46	51	23	40	8.6
	120	ND[a]	ND	ND	ND	
	180	ND	ND	ND	ND	
Cocaine and Ethanol						
	15	30	54	28	37	8.4
	30	390	289	280	320	35.3
	60	82	----	72	77	5.4
	90	61	111	44	72	20.1
	120	ND	ND	ND	ND	
	180	ND	ND	ND	ND	

[a] ND indicates compound not detected.

Table 3-30: Mean cocaine concentrations in SERUM (ng/ml) following an acute dose of cocaine (10mg/kg IP) with or without ethanol (2g/kg gavage).

	Time	Raw Data			Cocaine (ng/ml)	
	(min)	M1	M2	M3	Mean	SE
Cocaine only	15	ND[a]	5.4	1.2[b]	2.2	1.6
	30	8.9	7.2	1.3[b]	5.8	2.3
	60	2.4[b]	3.8[b]	1[b]	2.4	0.8
	90	1[b]	1[b]	1[b]	1	0
	120	ND	ND	ND	ND	
	180	ND	ND	ND	ND	
Cocaine and Ethanol						
	15	15.7	26.1	36.7	26.1	6.1
	30	15.7	15.2	6.4	12.8	3.2
	60	1[b]	1[b]	3.8[b]	1.9	0.9
	90	1[b]	1[b]	1[b]	1	0
	120	ND	ND	ND	ND	
	180	ND	ND	ND	ND	

[a] ND indicates compound not detected.

[b] Indicates concentation is below the LOQ and is therefore estimated.

Table 3-31: Mean BZE concentrations in SERUM (ng/ml) following an acute dose of cocaine (10mg/kg IP) with or without ethanol (2g/kg gavage).

	Time	Raw Data			BZE (ng/g)	
	(min)	M1	M2	M3	Mean	SE
Cocaine only	15	54	162	218	144	48
	30	195	180	258	211	24
	60	182	211	167	187	13
	90	102	145	110	121	12
	120	93	75	93	87	6
	180	73	30	80	61	16
Cocaine and Ethanol	15	138	134	134	135	1.4
	30	138	142	140	135	2.9
	60	130	86	102	106	13
	90	20	43	181	81	50
	120	25	43	143	70	37
	180	5	1[a]	15	7	4.2

[a] Indicates concentration is below the LOQ and is therefore estimated.

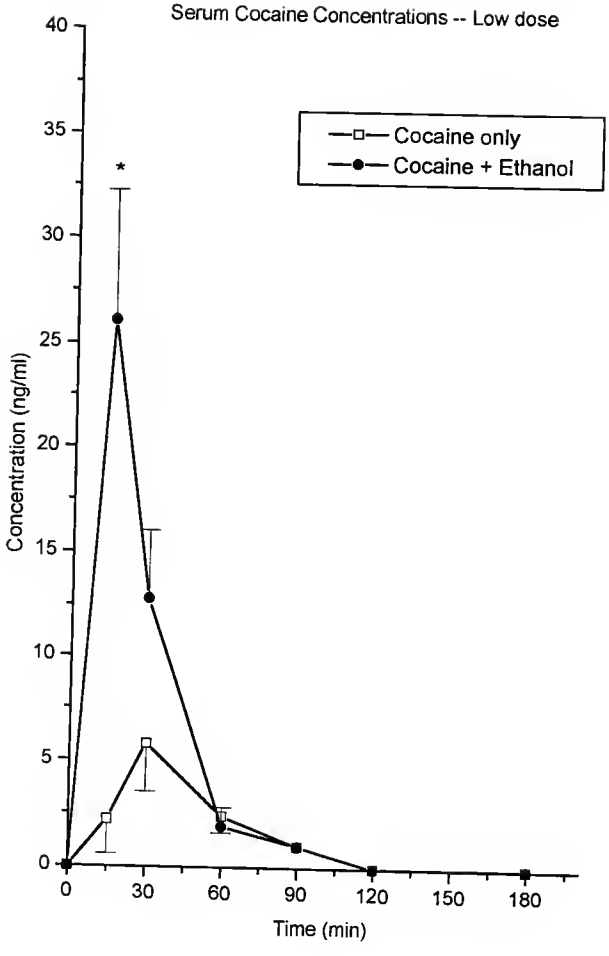

Figure 3-14: Serum cocaine concentrations in mice following a low dose of cocaine (10mg/kg) with or without an ethanol dose.

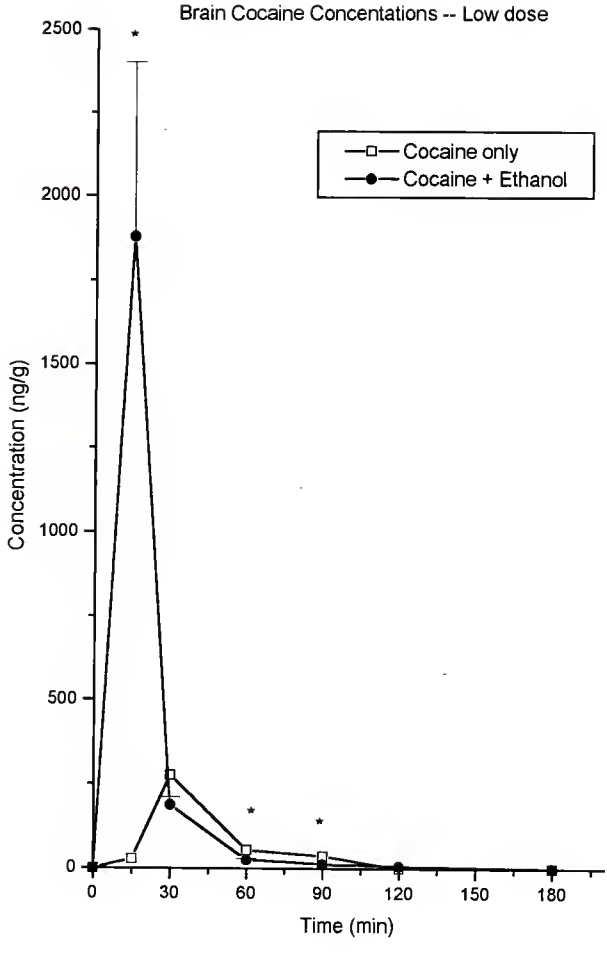

Figure 3-15: Brain cocaine concentrations in mice following a low dose of cocaine (10mg/kg) with or without an ethanol dose.

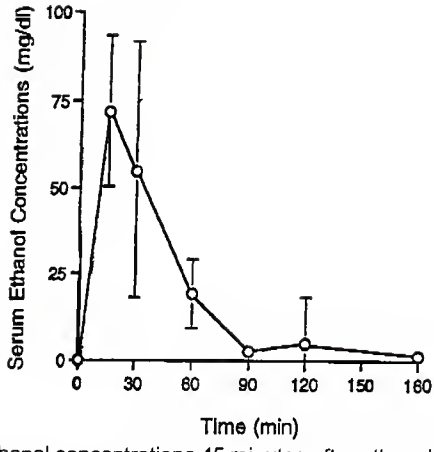

Figure 3-16: Serum ethanol concentrations 45 minutes after ethanol pretreatment.

In addition, serum ethanol concentrations were also measured in mice during the low dose cocaine experiment (Figure 3-16). Among mice pretreated with ethanol, the highest blood ethanol concentrations were measured at the first sampling time, which was 15 minutes after the first cocaine dose and 45 minutes after the ethanol pretreatment. Ethanol pretreatment increased the plasma AUC nearly 3-fold (cocaine alone: 265 ± 82 ng min ml^{-1}; cocaine + ethanol: 767 ± 49 ng min ml^{-1}; n=3; $p<0.05$). Peak serum BZE concentrations were decreased about one-third after ethanol pretreatment and BZE AUC also was decreased; however, this decrease was not significant (cocaine alone: 21900 ± 1100 ng min ml^{-1}; cocaine + ethanol: 14150 ± 3190 ng min ml^{-1}; n=3; $p<0.05$).

In brain, ethanol pretreatment resulted in significantly higher cocaine AUCs although brain cocaine concentrations were similar after 30 minutes (cocaine alone: 9400 ± 1100 ng min ml^{-1}; cocaine + ethanol: 33900 ± 8600 ng min ml^{-1}; n=3;

$p<0.05$). In contrast to the effect in serum, ethanol pretreatment significantly increased brain BZE concentrations and AUCs (cocaine alone: 5440 ± 410 ng min ml^{-1}; cocaine + ethanol: 13450 ± 1860 ng min ml^{-1}; n=3; $p<0.05$). To confirm this finding, the experiment was repeated and the same increase was observed (data not shown). In a follow-up experiment, mice were injected directly with BZE to evaluate its uptake into the brain.

Table 3-32: Mean BZE concentrations in BRAIN (ng/g) following an acute dose of BZE (10mg/kg IP) with or without ethanol (2g/kg gavage).

	Time	Raw Data			BZE (ng/g)	
	(min)	M1	M2	M3	Mean	SE
BZE only	15	429	338	287	351	42
	30	262	230	----[a]	246	13
	60	288	289	1412	663	100
	90	----	948	1145	1046	80
	120	153	749	453	452	172
	180	301	676	----	488	153
BZE and Ethanol						
	15	ND[b]	ND	ND		
	30	ND	ND	ND		
	60	ND	ND	ND		
	90	ND	ND	ND		
	120	ND	ND	ND		
	180	ND	ND	ND		

[a] ---- indicates sample not available for measurement at this time point.

[b] ND indicates compound not detected.

Table 3-33: Mean BZE concentrations in BLOOD (ng/ml) following an acute dose of BZE (10mg/kg IP) with or without ethanol (2g/kg gavage).

	Time	Raw Data			BZE (ng/ml)	
	(min)	M1	M2	M3	Mean	SE
BZE only	15	715	----[a]	382	548	136
	30	688	1236	950	958	158
	60	102	200	415	239	92
	90	217	136	250	201	34
	120	179	56	60	98	40
	180	30	40	30	33	3
BZE and Ethanol						
	15	412	307	541	420	68
	30	251	330	----	309**	32
	60	316	251	360	285	31
	90	244	298	----	271	22
	120	200	160	----	180	16
	180	----	100	115	107**	6

[a] ---- indicates sample not available for measurement at this time point.

** indicates statistical significance ($p<0.05$)

Cocaine and Ethanol Interactions in Pregnant Mice

Cocaine and ethanol interactions were also examined in pregnant mice in order to examine cocaine distribution to the fetus. Cocaine and benzoylecgonine concentrations were measured in fetal brain, maternal brain, and maternal blood. Cocaine was administered by IV injection to eliminate absorption variability.

Table 3-34: Cocaine concentrations in MATERNAL BLOOD (ng/ml) following an acute dose of cocaine (5mg/kg IV) with or without an ethanol dose (2g/kg gavage).

	Time	Raw Data				Cocaine (ng/ml)	
	(min)	M1	M2	M3	M4	Mean	SE
Cocaine only	15	27	39	30	----[a]	32	4
	30	4[b]	3[b]	4[b]	----	3	1
	60	ND[c]	ND	ND	----	ND	
	90	ND	ND	ND	----	ND	
Cocaine and Ethanol							
	15	123	92	144	111	118**	13
	30	29	29	32	44	34**	4
	60	ND	ND	ND	----	ND	
	90	ND	ND	ND	----	ND	

[a] ---- indicates sample not available for measurement at this time point.

[b] indicates concentration is below the LOQ and is therefore estimated.

[c] ND indicates compound not detected.

** indicates statistical significance ($p<0.05$)

Table 3-35: BZE concentrations in MATERNAL BLOOD (ng/ml) following an acute dose of cocaine (5mg/kg IV) with or without an ethanol dose (2g/kg gavage).

	Time	Raw Data				BZE (ng/ml)	
	(min)	M1	M2	M3	M4	Mean	SE
Cocaine only	15	229	110	87	----[b]	142	44
	30	124	154	125	72	144	14
	60	194	58	----	----	126	56
	90	ND[a]	ND	ND	ND	ND	
Cocaine and Ethanol							
	15	22	17	42	31	28**	6
	30	52	33	42	20	37**	8
	60	46	10	32	30	30**	9
	90	28	16	37	----	27**	6

[a] ND indicates compound not detected.

[b] ---- indicates sample not available for measurement at this time point.

** indicates statistical significance ($p<0.05$)

Table 3-36: Mean Cocaine concentrations in BRAIN (ng/g) following an acute dose of cocaine (5mg/kg IV) with or without ethanol (2g/kg gavage).

	Time	Raw Data				Cocaine (ng/g)	
	(min)	M1	M2	M3	M4	Mean	SE
Cocaine only	15-Fetal	1167	407	442	1003	755	194
	30-Fetal	354	414	368	----[b]	359	27
	60-Fetal	249	82	115	319	191	56
	90-Fetal	ND[a]	ND	ND	----	ND	
	15-Matern.	1843	2106	1280	968	1549**	259
	30-Matern.	374	329	374	893	492	134
	60-Matern.	88	41	83	127	83	18
	90-Matern.	ND	ND	ND	----	ND	
Cocaine and Ethanol							
	15-Fetal	418	690	545	916	642	107
	90-Fetal	424	381	361	460	407	22
	60-Fetal	155	255	95	615	280	116
	30-Fetal	83	142	95	----	87	30
	15-Matern.	1132	1282	846	1119	1095**	91
	30-Matern.	756	340	360	----	485	117
	60-Matern.	122	168	79	615	135	22
	90-Matern.	29	76	43	----	49	12

[a] ND indicates compound not detected.

[b] ---- indicates sample not available for measurement at this time point.

** indicates statistical significance ($p<0.05$)

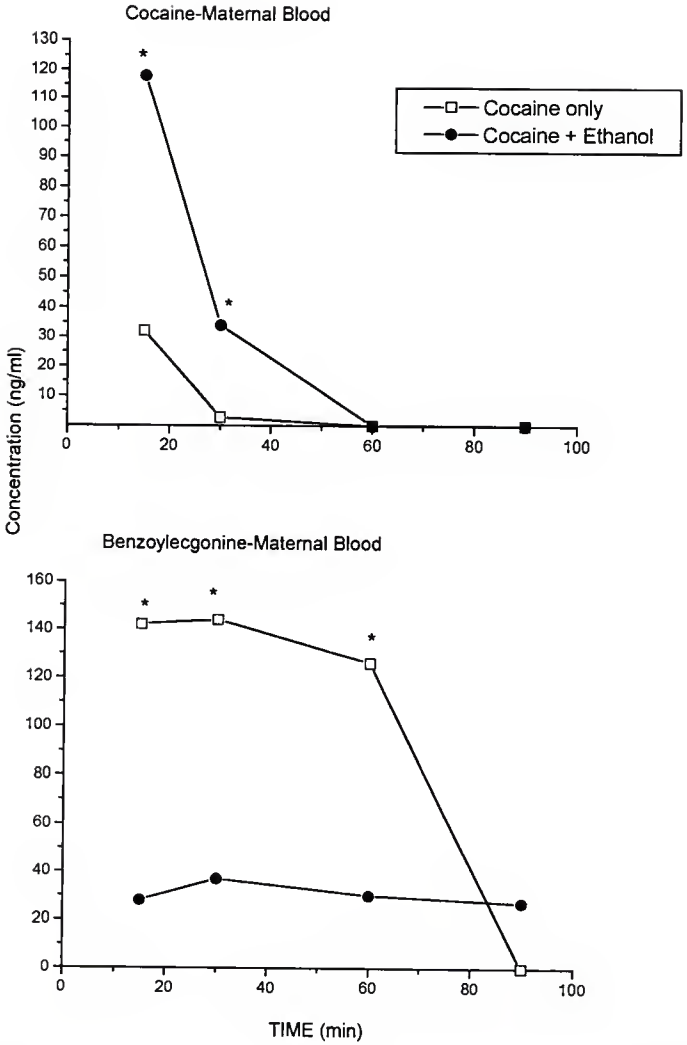

Figure 3-17: Maternal blood cocaine (top) and BZE (bottom) concentrations with and without an ethanol dose following an acute IV cocaine administration (5mg/kg).

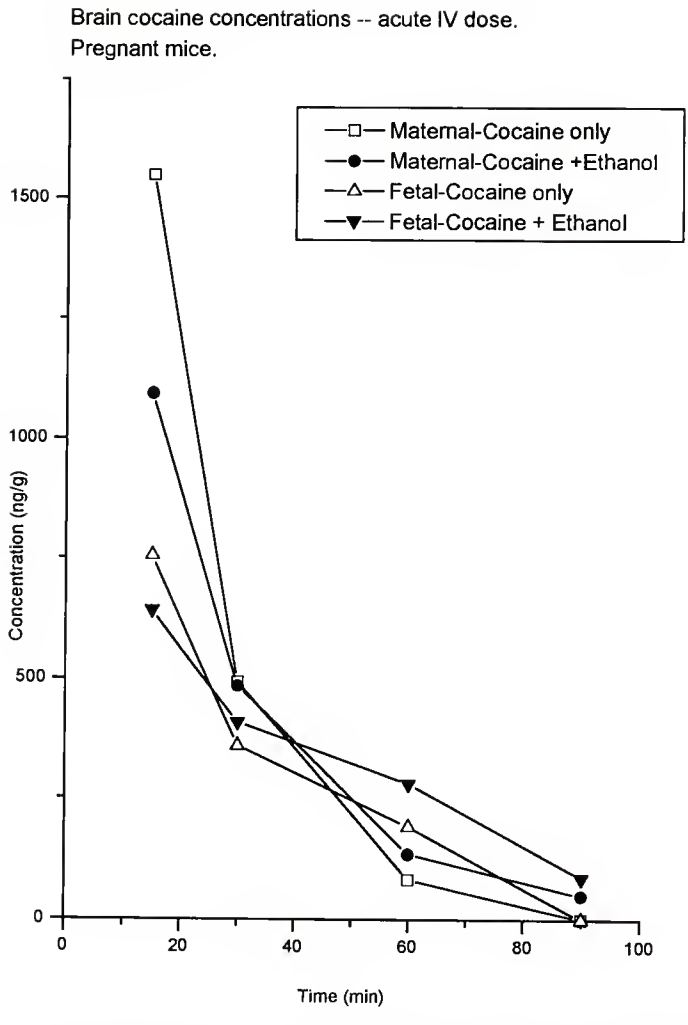

Figure 3-18: Maternal and fetal brain cocaine concentrations with and without an ethanol dose following an acute IV cocaine administration to the dam (5mg/kg).

Table 3-37: Apparent elimination rate and half-life for cocaine from mouse brain following acute cocaine IV administration (5mg/kg) calculated from a semi-log plot of the data in Figure 3-18.

Parameter	Maternal Cocaine Only	Maternal Cocaine+EtOH	Fetal Cocaine Only	Fetal Cocaine+EtOH
Ke (min)	0.0642	0.0411	0.0292	0.0251
T½ (min)	10.79	16.86	23.74	27.63
n	4	3	3	3
Maternal versus fetal cocaine only: ($p<0.0042$)[a]. Significant.				
Maternal versus fetal cocaine + EtOH: ($p<0.148$)[a].				
Maternal cocaine only versus maternal COC + EtOH: ($p<0.05$)[a]. Significant.				
Fetal cocaine only versus fetal COC + EtOH: ($p<0.35$)[a].				

[a] Significance testing by T-test.

Acute and Chronic Dosing of Cocaine and Ethanol in the Rat

Cocaine and ethanol interactions were also evaluted in rats following both an acute dose and a 24 day chronic dose schedule. Results indicate higher peak cocaine concentrations were achieved in the ethanol treatment group. These higher peak cocaine concentrations were also reached faster in this group.

Table 3-38: Mean drug concentrations in rat BLOOD (ng/ml) following an acute dose of cocaine (10mg/kg IP) with or without ethanol (1.5g/kg gavage).

	Time	BZE		Cocaine		Norcocaine		Cocaethylene	
	(min)	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Cocaine only	15	90	33	41	18	ND[a]		ND	
	30	125	49	270	252	ND		ND	
	60	118	49	125	67	ND		ND	
	90	111	31	22	6	ND		ND	
	120	91	25	22	7	ND		ND	
	180	59	22	21	5	ND		ND	
Cocaine and Ethanol									
	15	107	49	967	25	ND		ND	
	30	109	22	105	23	ND		ND	
	60	119	9	63	23	ND		ND	
	90	168	0	32	0	ND		ND	
	120	96	26	49	4	ND		ND	
	180	72	10	35	19	ND		ND	

[a] ND indicates compound not detected.

Table 3-39: Mean drug concentrations in rat BLOOD (ng/ml) following 24 day chronic dosing of cocaine (10mg/kg IP) with or without ethanol (1.5g/kg gavage).

	Time	BZE		Cocaine		Norcocaine		Cocaethylene	
	(min)	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Cocaine only	15	185	45	345	107	6	4	ND[a]	
	30	348	59	234	70	53	47	ND	
	60	194	56	130	39	89	61	ND	
	90	163	25	87	51	41	35	ND	
	120	117	4	87	48	32	32	ND	
	180	48	8	19	0	33	33	ND	
Cocaine and Ethanol	15	215	85	592	372	202	98	40	25
	30	261	75	378	160	132	98	40	21
	60	211	59	240	60	30	23	19	12
	90	200	52	198	57	37	27	24	14
	120	203	63	160	48	36	34	34	23
	180	111	55	159	63	57	66	ND	ND

[a] ND indicates compound not detected.

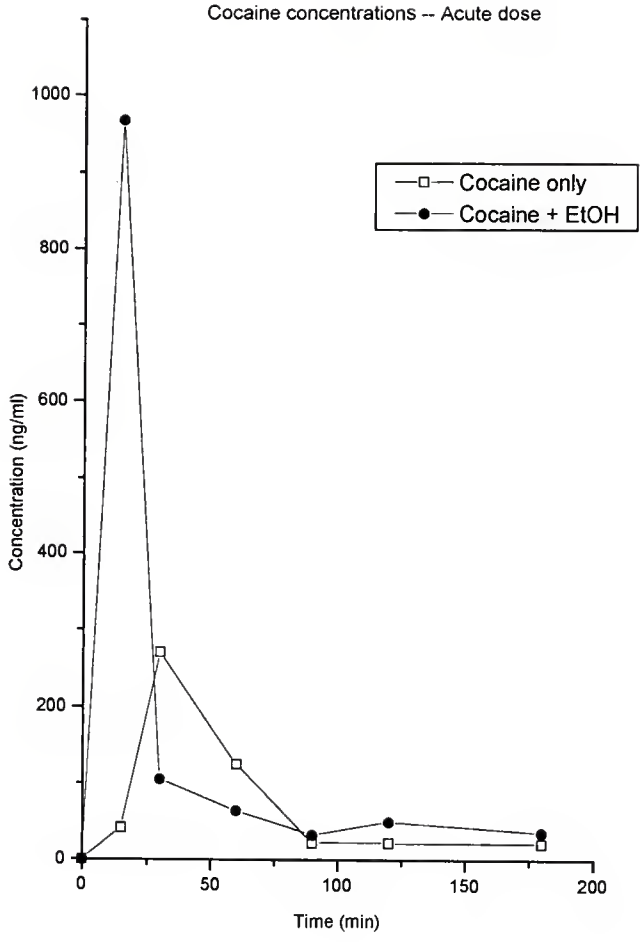

Figure 3-19: Cocaine concentration in rat blood following an acute dose of cocaine with or without ethanol.

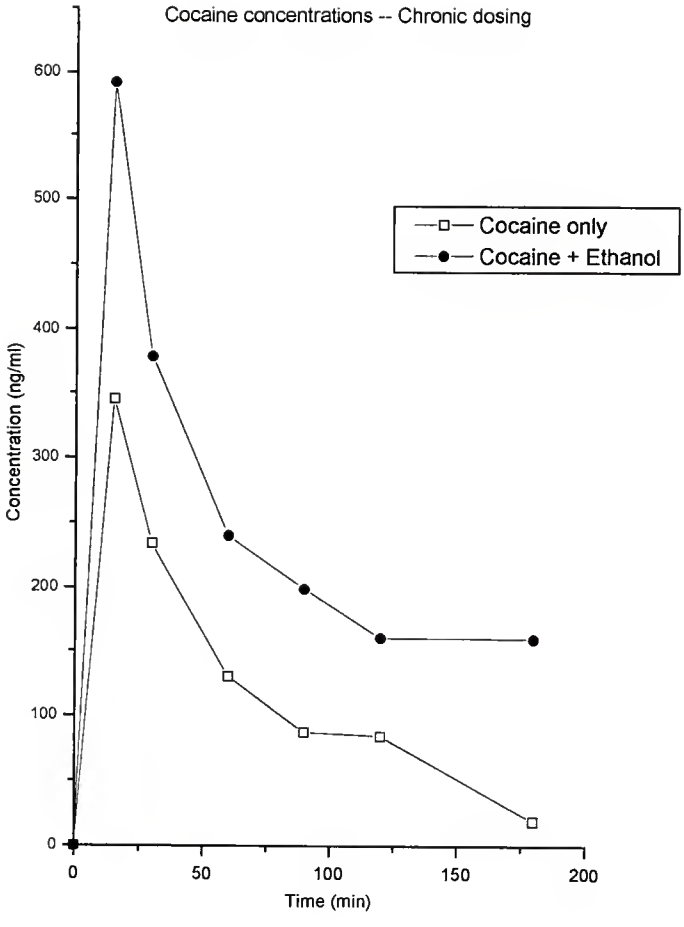

Figure 3-20: Cocaine concentration in rat blood following 24 day chronic dosing of cocaine with or without ethanol.

Table 3-40: Area-under-the-curve, Cmax, and Tmax for rat blood following acute as well as 24 day chronic dosing of cocaine (10mg/kg IP) with or without an ethanol dose (1.5mg/kg gavage). See Tables 40-41.

Sample Group	Drug	AUC	Tmax (min)	Cmax (ng/ml)
Chronic cocaine only	Cocaine	18840	15	345
Chronic cocaine/EtOH	Cocaine	36585**	15	592
Chronic cocaine only	BZE	22308	30	348
Chronic cocaine/EtOH	BZE	32451	30	261
Acute cocaine only	Cocaine	11505	30	270
Acute cocaine/EtOH	Cocaine	15191	15	967**
Acute cocaine only	BZE	15677	30	125
Acute cocaine/EtOH	BZE	14782	90	168

** Indicates significant difference ($p<0.05$)

Table 3-41: Mean drug concentrations in rat BRAIN (ng/g) following acute as well as 24 day chronic dosing of cocaine (10mg/kg IP) with or without an ethanol dose (1.5mg/kg gavage).

Sample Group	CPU[a] Cocaine	CPU Cocaeth	HYP[b] Cocaine	HYP Cocaeth
Chronic cocaine/EtOH	392	16	835	ND[c]
Chronic cocaine only	1258	ND	5277	ND
Acute cocaine only	2145	ND	4578	ND
Acute cocaine/EtOH	----[d]	----	----	----

[a] CPU indicates striatum
[b] HYP indicates hypothalamus
[c] ND = not detected
[d] ---- indicates sample not available

Specific Aim #4

Growth Pattern of IM-9 Cells

Initial experiments were performed to determine optimal conditions for cell growth using FBS concentrations of 1, 2, and 5% and cell densities of $1X10^4$, $2X10^4$, and $5X10^4$ cells/well. The combination of 2% FBS and a cell density of 2X10 cells/well provided optimal growth over 48 hours (Table 3-42).

A representative growth pattern of the IM-9 cells under incubation conditions is shown (Table 3-43). In serum-free medium, cell proliferation (as measured by thymidine incorporation) increased 13% from 24 to 48 hours. However, in 2% FBS media, proliferation increased almost 50% from 24 to 48 hours. IM-9 cell proliferation has previously been reported to be inhibited by treatment with phorbol 12-myristate 13-acetate [201]. With 40nM PMA, there was a statistically significant ($p< 0.05$) decrease in cell proliferation at both 24 and 48 hours. In serum-free medium, there was a 56% inhibition of proliferation compared to control with PMA at 24 hours which approached 72% inhibition by 48 hours. In 2% FBS medium, there was a 41% inhibition of proliferation at 24 hours which was maintained at 48 hours. Cell viability was calculated to be 92%$\pm$ 1.0% over all experiments.

Table 3-42: Initial experiments with IM-9 cells were performed to determine optimal conditions for cell growth.

Sample	Mean CPM 24 hour incubate	Std Error (CPM)		Mean CPM 48 hour incubate	Std. Error
SF grown $1x10^4$					
SF media	521	23		314	27
1% RPMI	3419	146		6369	423
2%	4166	199		9732	205
5%	4568	141		10568	374
Log phase $1x10^4$					
SF media	7005	1023		10046	601
1% RPMI	28804	1464		63718	1593
2%	25772	678		67308	2000
5%	25195	877		61885	3398
SF grown $2x10^4$					
SF media	3071	72		5074	109
1% RPMI	11544	318		20695	514
2%	12138	267		25547	593
5%	10778	319		29446	1140

Table 3-42--Continued.

Sample	Mean CPM 24 hour incubate	Std Error		Mean CPM 48 hour incubate	Std Error
Log phase $2x10^4$					
SF media	19890	620		33174	840
1% RPMI	44775	1039		100638	2239
2%	42148	1655		102406	2225
5%	43023	1431		93230	2388
SF grown $5x10^4$					
SF media	24375	579		34891	957
1% RPMI	39230	940		92272	2152
2%	40901	1302		96712	2860
5%	36157	1081		85265	2385
Log phase $5x10^4$					
SF media	68358	2286		62894	3440
1% RPMI	107679	2713		173040	3912
2%	99795	2178		180325	4499
5%	96054	2174		181755	6406

Table 3-43: Growth pattern of IM-9 cells. IM-9 cell proliferation over 48 hours is shown for a typical experiment. The effect of the PMA (40nM) negative control is also presented from the same experiment.

Incubation	- PMA CPM ± SE	+ PMA CPM ± SE
Serum-free		
24 hours	30,382 ± 434	13,361 ± 269
48 hours	34,895 ± 1,891	9,886 ± 252
2% FBS		
24 hours	38,114 ± 649	22,335 ± 342
48 hours	71,582 ± 2,204	36,976 ± 647

Proliferation Assays

Cocaine

Incubation of IM-9 cells with cocaine or its metabolites demonstrated significant increases in cell proliferation. Incubation with cocaine demonstrated statistically significant increases in proliferation (110 -148% of control CPM) at almost all concentrations and incubation conditions. Maximal stimulation of 148% occured in the 48 hour serum-free medium incubation at 1.0ug/ml. Dose-response curves for cocaine showed a dose-response relationship in the 48 hour incubation with peak proliferation at 1.0 ug/ml in serum-free medium and at 0.1 ug/ml in 2% FBS medium. No significant dose-response relationship was observed in the 24 hour incubation (Figure 3-21).

Benzoylecgonine

IM-9 cell incubation with benzoylecgonine resulted in statistically significant increases in cell proliferation in both serum-free medium and 2% FBS medium at 48 hours with proliferation of 109-170% of control CPM. At 24 hours, there was a slight, but statistically significant, stimulation in serum-free medium at drug concentrations of 0.1 ug/ml or greater. No effect was seen in 2% FBS medium at 24 hours. Dose-response curves showed increasing stimulation in serum-free medium at 48 hours, but no significant dose-relationship was observed in 2% FBS medium. At 24 hours, peak stimulation of 170% was observed at 0.1 ug/ml benzoylecgonine (Figure 3-22).

Norcocaine

IM-9 cell incubation with norcocaine demonstrated statistically significant stimulation of proliferation only in serum-free medium with stimulation of 114-150% of control CPM. This effect was seen at both 24 and 48 hours. Maximal stimulation of 121% of control CPM at 24 hours and 150% at 48 hours occured at 0.1 ug/ml. No effect of norcocaine was observed in 2% FBS medium. Dose-response curves showed bell shaped relationships between drug concentration and percentage stimulation in serum-free medium at both 24 and 48 hours (Figure 3-23).

Cocaethylene

Incubation with cocaethylene demonstrated statistically significant increases in cell proliferation only in serum-free medium with maximal stimulation at the highest drug concentration (10 ug/ml). At 24 hours, proliferation was stimulated at cocaethylene concentrations of 0.1ug/ml or greater reaching maximal stimulation

of 116%. At 48 hours, proliferation was stimulated at concentrations of 1.0 and 10ug/ml with maximal stimulation of 146%. Dose response curves for cocaethylene illustrate no relationship at 24 hours, but an increase in proliferation in serum-free medium at 48 hours (Figure 3-24).

Drug Stability

HPLC analysis of cocaine, cocaethylene, norcocaine, and benzoylecgonine indicated that these drugs are stable when incubated in medium for 48 hours (data not shown). Benzoylecgonine showed no significant changes. Norcocaine and cocaethylene showed an approximate 10% degradation to norbenzoylecgonine and benzoylecgonine, respectively, over 48 hours. Cocaine showed an approximate 25% hydrolysis to benzoylecgonine over 48 hours. This pattern of drug stabilities was not altered by either the presence of 2% FBS or the presence of IM-9 cells.

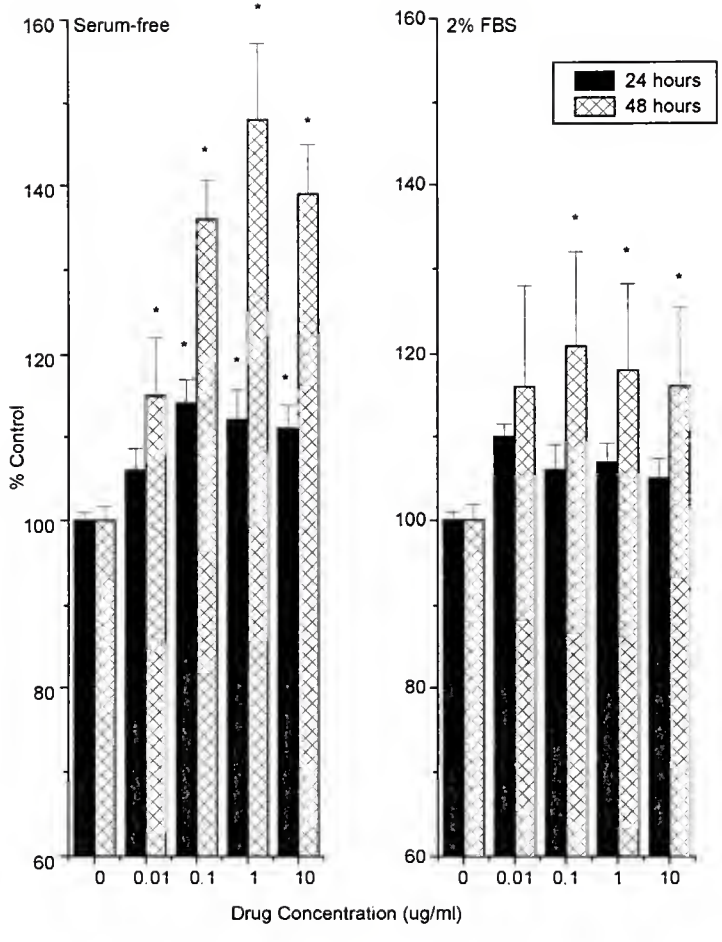

Figure 3-21: Cell proliferation when incubated with cocaine in RPMI 1640 serum-free media (left panel) and 2% FBS media (right panel) for 24 and 48 hours. Control CPM mean values: 24 hour serum-free = 41227 CPM, 48 hour serum-free = 38864 CPM, 24 hour 2%FBS = 62053 CPM, and 48 hour 2%FBS = 119838 CPM.
* = $p<.05$

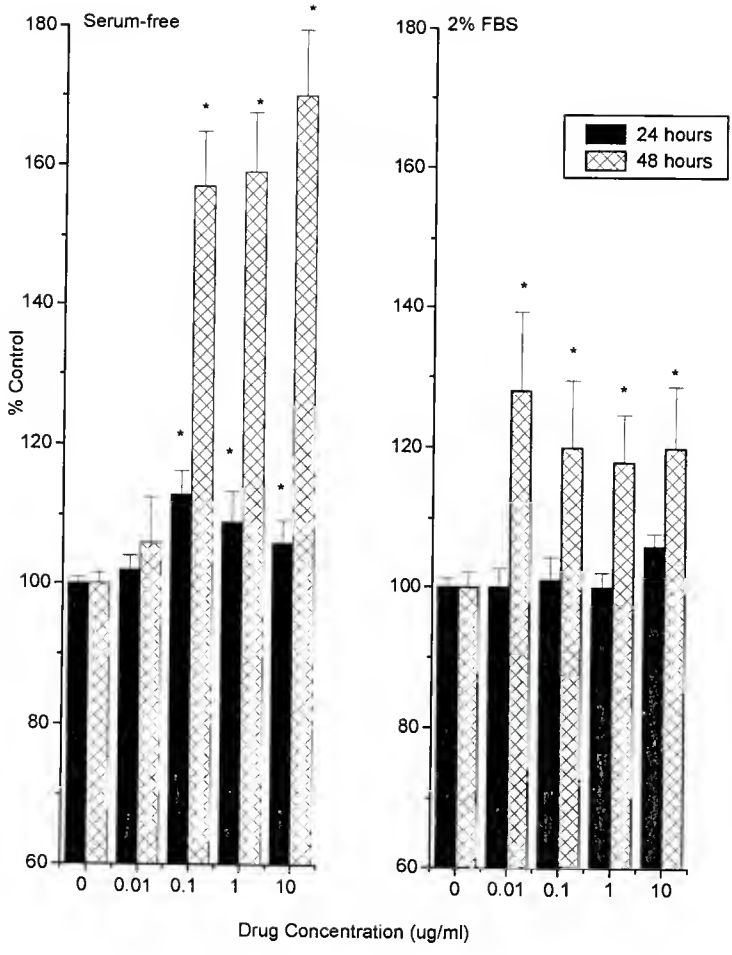

Figure 3-22: Cell proliferation when incubated with benzoylecgonine in RPMI 1640 serum-free media (left panel) and 2% FBS media (right panel) for 24 and 48 hours. Control CPM mean values: 24 hour serum-free = 41227 CPM, 48 hour serum-free = 38864 CPM, 24 hour 2%FBS = 62053 CPM, and 48 hour 2%FBS = 119838 CPM.
* = $p<.05$

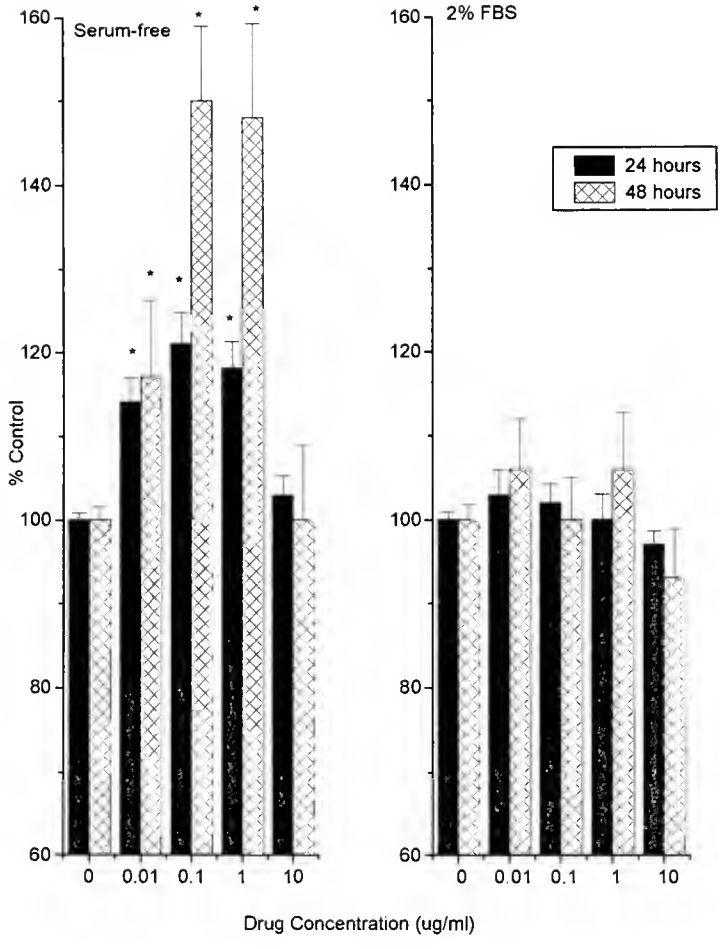

Figure 3-23: Cell proliferation when incubated with norcocaine in RPMI 1640 serum-free media (left panel) and 2% FBS media (right panel) for 24 and 48 hours. Control CPM mean values: 24 hour serum-free = 41227 CPM, 48 hour serum-free = 38864 CPM, 24 hour 2%FBS = 62053 CPM, and 48 hour 2%FBS = 119838 CPM.
* = $p<.05$

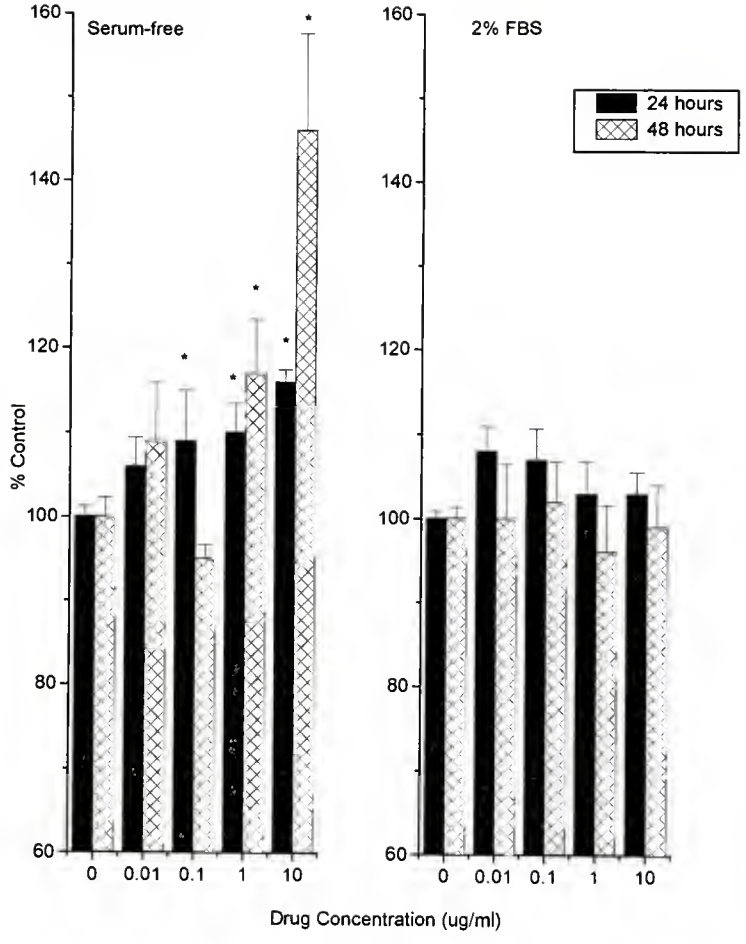

Figure 3-24: Cell proliferation when incubated with cocaethylene in RPMI 1640 serum-free media (left panel) and 2% FBS media (right panel) for 24 and 48 hours. Control CPM mean values: 24 hour serum-free = 41227 CPM, 48 hour serum-free = 38864 CPM, 24 hour 2%FBS = 62053 CPM, and 48 hour 2%FBS = 119838 CPM.
* = p<.05

Cord Blood PBMCs Isolated from Cocaine Users

The proliferative response of cord blood PBMCs isolated from both cocaine users and non-users were evaluated using phytohemagglutinin (PMA). There was considerable variability between individuals in their response to the mitogen (Table 3-44). Statistical analysis was done by comparison of the mean values of the cocaine target group versus the control group.

Table 3-44: Proliferative response of cord blood PBMCs (from both cocaine users and non-cocaine users) after incubation with PHA for 72 hours. The background CPM's are subtracted from the stimulated cell CPMs for final analysis.

Subject group	Stimulated Cells		Unstimulated cells	
	Mean (n=3)	SE	Mean (n=3)	SE
Cocaine Target	89600	38692	1259	411
Control	40823	6008	498	269
Cocaine Target	31740	1428	151	22
Control	71678	12358	1067	60
Cocaine Target	141025	7367	742	150
Control	25754	6057	588	135
Cocaine Target	852	115	317	135
Control	1309	364	682	144
Cocaine Target	94555	7177	1650	177
Control	141477	14441	2214	397

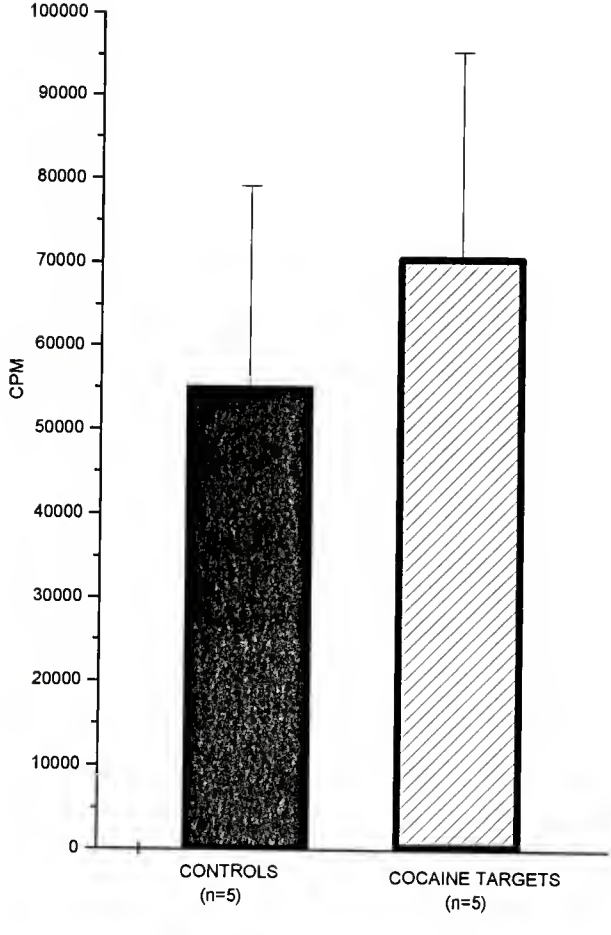

Figure 3-25: Mean proliferative response to PHA (in CPM) for cocaine target group compared to the drug free control group for cord blood lymphocytes.

CHAPTER 4
DISCUSSION

This research project was designed to investigate the following hypotheses: (1) the fetus is exposed to higher concentrations of cocaine than is the mother during maternal cocaine use in pregnancy; (2) the concurrent use of cocaine with ethanol increases cocaine concentrations; and, (3) cocaine and its metabolites have an adverse effect on the immune system. In order to investigate these hypotheses, it was necessary to first develop and validate analytical methods for the detection and quantitation of cocaine and metabolites in a variety of biological matrices from both humans and animals. Analytical methods utilized for satisfying the specific aims of this research included fluorescence polarization immunoassay (FPIA), solid-phase extraction (SPE), high performance liquid chromatography (HPLC), gas chromatography-mass spectrometry (GC-MS), tissue culture and liquid scintillation counting.

Solid-phase extraction was used to extract cocaine and metabolites from the biological matrices. Although solid-phase extraction has been described for the extraction of cocaine and metabolites from plasma and urine, this work represents the validation of this technique in combination with chromatographic methods of analyses for the determination of cocaine in a range of biological samples. These

include: urine, whole blood, cord blood, serum, meconium, amniotic fluid, brain, liver, and placenta. This method was selected over liquid-liquid extraction because it is much more rapid, requires less solvent, and is generally more efficient. Solid-phase extraction is performed on bonded silica phases with the interaction dependent on the type of bonded functional group. Sorbents selected for cocaine and metabolites were nonpolar sorbents with long-chain hydrocarbons (C2, C8, C18) attached to the silica (C2 being the most polar). In addition, a strong cation exchange (SCX) sorbent was used because in addition to its nonpolar interaction the propylbenzoylsulfonyl functional group bonded to the silica also can be used to extract molecules carrying a positive charge (such as cocaine at low pH). The percentage of drug recovered following extraction is reported in Tables 3-6 and 3-7. Solid-phase extraction resulted in good to excellent recovery for all compounds from all biological matrices with recoveries of 62-96%. Recoveries reported were: meconium 81- 91%, urine 84%-93%, amniotic fluid 62-86%, whole blood 75-91%, cord blood 80-88%, serum 83-96%, and brain 81-90%. The lowest recovery reported from all biological matrices was for benzoylecgonine and the highest was for cocaethylene.

The primary analytical method used for detection and quantitation of cocaine and metabolites was HPLC. Interday variability of cocaine and metabolite concentrations ranged from 2.9-13.9% and intraday variability was 1.1-3.1%. The linearity and precision of calibration curves were monitored over the course of the study and demonstrated excellent reproducibility. Method validation studies

performed using HPLC system 2 (which utilizes a tunable absorbance detector operated at 230nm and Waters Millennium chromatography management software) provided confidence in the reproducibility of the quantitation over the course of the study. While GC/MS is commonly accepted as the "gold standard" analytical technique for the determination of drugs of abuse, this work relied heavily on the use of HPLC. Results of the experiment to compare HPLC analysis of cocaine and metabolites to GC/MS indicate that HPLC is a valid alternate method of analysis for certain analytes. Sensitivity of the analytical methods for each compound was compared by paired t-tests on the mean slope determinations reported from 3 separate calibrations determined by each chromatographic method. Although GC/MS yielded the best sensitivity for cocaine, HPLC showed greater sensitivity for norcocaine, and the 2 methods were equivalent for benzoylecgonine and cocaethylene (at the level of $p<0.05$). The limit of detection and limit of quantitation were determined for each compound with the LOD for HPLC of 1ng/ml for BZE, cocaine, and norcocaine and 2-4ng/ml for cocaethylene. The LOD for GC/MS was 1ng/ml for BZE and cocaethylene, 2ng/ml for cocaine, and 10ng/ml for norcocaine. Precision was compared by paired t-tests($p<0.05$) on the interday variabilities with GC/MS demonstrating the best precision (or lowest interday variability) for all compounds except cocaine (6.99% for HPLC versus 8.28% for GC/MS). Interday variabilities for this experiment were: HPLC (3.79-7.18%) and GC/MS (1.98-8.28%). However, norcocaine was not reproducibly nor completely derivatized and would require daily calibration for accurate quantitation by GC/MS. An alternative

derivatization procedure has been reported for norcocaine using HFIP (hexafluoroisopropanol) instead of PFPOH (pentafluoropropanol) which may circumvent this problem. Quantitation of benzoylecgonine in human urine samples by both methods demonstrated excellent linear correlation between HPLC and GC/MS (r=0.9992). While it may be surprising that HPLC provides comparable, and in some situations superior, results when compared to GC/MS, similar results were reported from the analysis of sertraline, an antidepressant, using both GC/MS and HPLC [202]. Once validated, these analytical methods were then used for sample analysis in support of the specific aims of the project.

Due to the documented adverse effects of gestational cocaine exposure discussed in Chapter 1, acquiring sensitive methods of identification of gestational cocaine exposure in humans was one of the initial aims of this project. In addition, another early objective was to evaluate whether the procedures routinely employed by hospital laboratories to identify cocaine exposed neonates are adequate for the task. In general, hospitals do not screen all neonates or their mothers for detection of gestational cocaine use. Clinicians and researchers at many hospitals have completed large scale anonymous screening of urine samples in order to determine the prevalence of cocaine use in their patient population; however, these studies are usually completed within a specific time period after which only a select number of patients are tested. The procedure utilized by hospital laboratories for identification of gestational drug use involves screening of urine (either urine from the mother, on admission for labor and delivery, or neonatal urine collected in the

first 24 hours of life) by immunoassay. Immunoassay is the preferred technique because it provides rapid, sensitive screening for a variety of drugs of abuse. It is also cost effective for the large scale screening of urine samples. The same urine sample can be used to screen for cocaine metabolite, opiates, amphetamines, barbiturates, and benzodiazepines. Another advantage of immunoassay over chromatographic methods of analysis is that the drug does not need to be extracted from urine prior to analysis. Immunoassay screening for cocaine use involves reaction of the antibody with benzoylecgonine, a major urinary metabolite. All immunoassay techniques are targeted on benzoylecgonine and most demonstrate extremely limited cross-reactivity with cocaine or ecgonine methyl ester. This limitation may be a significant problem in the identification of gestational cocaine exposure due to recent evidence indicating that the developing fetus has a reduced capacity to metabolize cocaine to benzoylecgonine [8,192]. Dusick et al [8] reported that while cocaine and norcocaine were detected in the meconium of a cohort of very low birthweight babies (<1500 grams), no benzoylecgonine was detected. This observation signified lack of metabolism by the fetus. In that study, immunoassay of neonatal urine identified only 8% of the neonates eventually assigned to the cocaine exposed group while 90% were identified from a combination of maternal history and/or meconium testing.

Therefore, given the information indicating the presence of unmetabolized cocaine in the meconium of neonates exposed to cocaine during gestation and the need for a rapid method of toxicologic testing needed for screening large numbers

of samples, experiments were designed in an effort to improve immunoassay detection of samples containing cocaine. In these experiments, the goal was to determine if unmetabolized cocaine in neonatal urine and meconium could be hydrolyzed to benzoylecgonine to permit the subsequent detection of benzoylecgonine by immunoassay. Initial experiments (Tables 3-1 to 3-3) found that cocaine spiked into human urine could be completely hydrolyzed to benzoylecgonine. This was done by adjusting the pH of the urine sample to a pH>9.5 with concentrated NaOH, incubating the sample for 30 minutes at 50°C, and readjusting the pH of the sample to pH8.0 with HCl in preparation for screening by FPIA.

When this method was applied to clinical samples obtained from neonates for toxicology testing, however, the method did not improve the detection of gestational cocaine exposure. Only 1 urine sample and 1 meconium sample yielded a higher concentration of benzoylecgonine following hydrolysis and these concentrations were still below the cut-off limit (Tables 3-4 and 3-5). It was also determined that the procedures followed in an effort to hydrolyze cocaine to BZE were as time-consuming as analysis of the same samples by chromatographic methods and would not, therefore, provide the rapid method needed by a hospital clinical laboratory for screening large numbers of samples. However, results indicate that immunoassay screening of amniotic fluid may be beneficial in identifying cocaine exposure since it would appear that this medium contains high concentrations of BZE. In cocaine using women, this may reflect sequestration of

the drug since BZE does not readily cross the placental barrier due to its low lipophilicity and will therefore remain on the fetal side of the placenta. In addition, it has also been suggested that cocaine accumulates in the fetus via the process of ion trapping due to the lower pH of fetal blood and amniotic fluid as compared to maternal serum [15]. The Henderson-Hasselbalch equation, $pH-pKa = \log [B/BH^+]$, describes how the ionization of a drug is dependent on the pKa of the drug and the pH of the solution in which it is dissolved. Cocaine is a weak base with a pKa of 8.5. Maternal serum has a pH of 7.4 and the pH-pKa difference for cocaine is therefore -1.1 indicating 92.06% of the drug is ionized. However, in fetal blood (pH 7.28), the pH-pKa difference is -1.22 and 93.97% of the drug would be ionized. In amniotic fluid (pH 7.0-7.25), the pH-pKa difference is -1.25 to -1.5 and the fraction ionized therefore rises to 94.38-96.84%. The increased ionization of cocaine in the lower pH environment of amniotic fluid would lead to its accumulation in that environment.

Fourteen paired amniotic fluid and newborn urine samples were screened by AD_x^{TM} during the course of this project prior to subsequent analysis by HPLC. Results indicate that while immunoassay identified fewer positive amniotic fluid samples than did HPLC analysis of the same samples, the percentage of amniotic fluid samples identified as cocaine exposed was higher than that of the corresponding infant urine. Several of the amniotic fluid samples screening negative by immunoassay, but positive by HPLC were characterized as being particularly viscous, discolored, and/or bloody which may have resulted in the failure of the

antibody to react with the drug. The amniotic fluid samples were screened by the AD_x^{TM} kit designed for urinalysis. Ripple at al [199] reported screening of 450 amniotic fluid samples obtained during amniocentesis by FPIA. While the incidence reported in their population was low (1.3%), FPIA analysis of amniotic fluid spiked with benzoylecgonine was very sensitive and accurate with an average percent error of BZE concentration in spiked amniotic fluid of 8%. Results indicate hospital laboratory screening of amniotic fluid, rather than newborns' urine, may provide the rapid, sensitive, cost effective method needed for identification of gestational cocaine exposure. The major disadvantage to using amniotic fluid, however, is that this sample is not routinely collected during labor and delivery. There is also difficulty observed in amniotic fluid collection from some women in labor either due to their arrival at the hospital after membrane rupture or to their delivering by cesarean section (Personal communication, Kathie Wobie, M.S., Clinical Research Coordinator, University of Florida).

As mentioned previously in this discussion, most hospitals do not screen all newborns or their mothers for detection of gestational cocaine use, but physicians and nurses select individuals for testing based on individual factors. The aim of this part of the project was to determine the sensitivity of comprehensive meconium testing by HPLC as compared to a protocol of targeted urine screening by immunoassay (EMIT) in the detection of prenatal cocaine exposure from neonates. Meconium was collected from all neonates delivered in a one month period at both an urban community hospital and a suburban community hospital. Neonates and

their mothers were "targeted" for urine testing based on demonstrated clinical risk factors for cocaine use by the mother. During the study period, 312 neonates were enrolled and 62 were identified as cocaine exposed. Unmetabolized cocaine was detected in 75% of the meconium samples from the cocaine exposed group with concentrations of 140-3498ng/g. While benzoylecgonine was present in 69% of the meconium samples from the cocaine exposed group (92-8433ng/g), the detection of cocaine, and of such high concentrations of cocaine, in meconium provides evidence that the fetus does not metabolize cocaine to the same extent as adults. When incidence identification and method sensitivity were compared, it was found that comprehensive meconium testing provided significantly better incidence identification than targeted urinalysis (17% versus 3.2%) and significantly higher test sensitivity (85.5% versus 16.1%). In addition, when the entire group of mothers and neonates was evaluated for the presence of clinical risk factors, while the cocaine exposed group had a higher percentage demonstrating at least 1 risk factor, it was found that 69% of this group presented none of the risk factors used to target individuals for urinalysis. Results indicate that the procedures employed by many hospitals to identify cocaine exposed neonates are inadequate since most use a "targeting" protocol similar to that used for this study and/or immunoassay procedures which, as previously described, are unsuitable for premature babies. These results also would signify that the control groups in some clinical studies evaluating the effects of gestational cocaine exposure may be compromised and include neonates from women who used cocaine during their pregnancy. The

procedures used to "target" the cocaine group are biased and raise questions about previous reports of the clinical effects of gestational cocaine use

The next aim of this project was to characterize maternal/fetal cocaine metabolism and distribution by testing the hypothesis that the fetus is exposed to higher concentrations of cocaine than is the mother during maternal cocaine use in pregnancy. The results partially support this hypothesis. Results of the analysis of paired samples of neonatal urine and meconium (Table 3-19) agree with previously published studies suggesting that meconium is the better matrix for detection of gestational cocaine exposure. The concentrations of cocaine and metabolites present were quite high in some cases (>8000ng/g) indicating the fetus was exposed to high cocaine concentrations during fetal life. In the study evaluating comprehensive meconium testing versus targeted urine screening, 23% of the meconium samples from the cocaine exposed group contained norcocaine (trace-1357ng/g) and 68% contained benzoylecgonine (92-8433ng/g). The formation of norcocaine is a minor pathway of cocaine metabolism in the adult. The presence of norcocaine would perhaps indicate the fetus expresses a different population of the cytochrome P450 3A family responsible for the oxidative metabolism of cocaine. The production of norcocaine is also reported to be higher in individuals with lower cholinesterase activity and it has been shown that the developing fetus has lower cholinesterase activity [65]. The presence of BZE indicates that, unlike premature low birthweight babies, the term neonate has the

capacity to metabolize cocaine to BZE. This data would indicate the capacity of the fetus to metabolize cocaine evolves during the gestational period.

The human study using biological fluids from 14 women with a history of cocaine use during pregnancy (Table 3-21), shows the highest concentrations of BZE (a major cocaine metabolite in adults) are found in the amniotic fluid. Matched samples of cord blood, amniotic fluid, and neonatal urine were collected and analyzed by HPLC following SPE. Fourteen amniotic fluid samples were analyzed and 10 were positive for BZE (trace-3100ng/ml). Twelve neonatal urines were available for analysis, and 5 were positive for BZE (100-1100ng/ml). Of the 8 cord blood samples available, 6 were positive for BZE (120-770ng/ml) indicating recent use of cocaine by the mother. Three mother/neonate pairs had a negative urinalysis, but the corresponding amniotic fluid samples were positive. In addition, unmetabolized cocaine was detected in 4 of the amniotic fluid samples. Since the fetus is continually exposed to amniotic fluid throughout gestation, the presence of high concentrations of cocaine metabolite in the fluid is of concern as it exposes the developing fetus to the toxic effects of the drugs for a much longer period of time than the mother.

One animal study was completed to evaluate drug distribution to the fetus. In the study, female, timed-pregnant ICR mice were injected with a single dose of cocaine with or without a pretreatment of ethanol on day 17 of gestation. In this study, the cocaine was administered intravenously via a tail vein to eliminate drug absorption as a variable. Mice were sacrificed at time points up to 90 minutes after

the cocaine dose was administered and maternal blood and maternal and fetal brains were collected. Again, results show that cocaine rapidly crosses to the fetuses following cocaine administration to the mother. While initial cocaine concentrations were significantly higher in maternal brain than in fetal brain, fetal concentrations of cocaine were essentially equivalent to maternal concentrations by 30 minutes after the cocaine dose, and by 60 minutes, fetal concentrations were higher than maternal. Using a semi-log plot of the brain concentrations, the apparent elimination rate and half-life of cocaine in the brain was calculated. It was found that there was a statistically significant difference in elimination half-life from maternal and fetal brain after an acute cocaine dose (maternal = 10.79 minutes and fetal = 23.74 minutes).

Results of these experiments provide evidence that while the fetus does receive high doses of cocaine after drug administration to the mother, concentration differences are dependent on time. These results are consistent with other reports which show initially higher maternal cocaine concentrations, but subsequently higher or comparable fetal concentrations [85,87,89,91,92]. The placenta plays an important role in drug elimination from the fetus; therefore, it is possible that while the developing fetus has immature drug metabolism and elimination capabilities when compared to the adult, the placenta provides a means of protecting the fetus from extremely high drug concentrations. Experimental evidence also has demonstrated decreased uterine blood flow and placental insufficiency following cocaine administration which may also serve to reduce the amount of drug crossing

the placenta. Reports of alterations in fetal cerebral blood flow [109-110] are also significant to this discussion due to the interest in drug distribution to the fetal versus maternal brain. Blood flow alterations induced by cocaine administration to the mother could potentially alter drug distribution in the fetus. Burchfield et al [110] report increased cerebral blood flow in the fetus in response to maternal cocaine administration; however, no information was available regarding alterations in blood flow to the remaining fetal body. Fetal hypoxemia has been observed in response to cocaine administration to the mother. The fetal response to hypoxemia is generally an increased cardiac output and increased blood flow to the brain and myocardium to maintain oxygen delivery [203]. It may be that cerebral blood flow is increased at the expense of the fetal body. For future research, it would be worthwhile to compare fetal brain to whole fetus cocaine concentrations following maternal administration.

Based on the results of the cocaine distribution studies in this project and literature reports of the number of cocaine users also using ethanol, the second hypothesis of this study was formulated: the simultaneous use of ethanol and cocaine significantly alters the metabolism of cocaine thereby prolonging the duration of action and increasing cocaine concentrations. The specific aim of this aspect of the project was an *in vivo* examination of the effect of ethanol cotreatment on blood and brain cocaine and benzoylecgonine concentrations in mice. Recent *in vitro* studies with both mouse and human liver have indicated that ethanol can significantly alter the metabolism of cocaine by inhibiting the formation of

benzoylecgonine [162-164]. Inhibition of the carboxylesterase metabolism of cocaine to benzoylecgonine would, therefore, be expected to increase cocaine concentrations in blood and tissues. Cocaine concentrations in the brain are of particular interest given reports by cocaine users that the cocaine/ethanol combination enhances the "high" experienced with cocaine use. In order to evaluate these questions, a series of experiments was conducted in rats and mice.

In the first study, male ICR mice were injected with a sub-lethal dose of cocaine (55mg/kg IP) with or without ethanol pretreatment (3g/kg gavage). These extremely high doses were chosen for the initial work to also examine the potential increased mortality risk with ethanol. Brain and serum cocaine and metabolite concentrations were measured. Serum ethanol concentrations were also determined (75mg/dl on average). Results (Tables 3-22 to 3-27) show that very high concentrations of cocaine and metabolites were quantitated in both serum and brain. Mean peak serum cocaine concentrations (n=3 mice) were 2.5 times higher and mean BZE concentrations were lower (and reached peak concentration later) in the mice also receiving ethanol. Small amounts of cocaethylene were detected in serum at the earliest time points (92-157ng/ml). In brain tissue, mean peak cocaine concentrations were almost 2 times higher in the mice also receiving ethanol; however, BZE and norcocaine concentrations did not differ significantly. Cocaethylene was detected in brain tissue up to 90 minutes after a cocaine dose, yet considering the extremely high dose of cocaine administered, these concentrations were not particularly high (184-776ng/g). In addition, there was no

increased mortality observed in the mice receiving ethanol. These findings support the hypothesis that ethanol/cocaine interaction increases cocaine concentration; however, the concentrations were far higher than those seen in humans after cocaine use. For this reason, a second study was completed and was designed to produce plasma concentrations relevant to human cocaine use.

In the second study, male ICR mice were injected with cocaine (10mg/kg IP) with or without ethanol pretreatment (2g/kg gavage). In serum, ethanol pretreatment resulted in an approximately 5-fold increase in the peak cocaine concentration and in a statistically significant increase in the plasma cocaine AUC. Mean peak plasma BZE concentrations were reduced by ⅓ and there was a slight, but statistically insignificant decrease in the plasma BZE AUC with ethanol pretreatment. In brain tissue, ethanol pretreatment resulted in an approximately 6-fold increase in peak cocaine concentration and a statistically significant increase in the brain cocaine AUC. The brain cocaine AUCs were significantly different even though, by 30 minutes after the cocaine dose, the concentrations were similar between the cocaine only and the cocaine/ethanol groups. Somewhat surprisingly, ethanol pretreatment resulted in an approximate 4-fold increase in mean peak BZE concentration and a statistically significant increase in brain BZE AUC. No cocaethylene was detected in these samples which would indicate that very high cocaine and ethanol concentrations are needed for formation of this metabolite.

The changes in serum cocaine and benzoylecgonine concentrations produced by ethanol pretreatment are consistent with an inhibition of cocaine

esteratic metabolism and support hypothesis 2 for this project. Increases in peak cocaine concentration in serum were coupled with increases in peak brain cocaine concentrations. The observed increases in brain cocaine concentration provide a mechanism by which the concurrent use of ethanol with cocaine might increase the behavioral effects and CNS related events. The increased brain BZE is of some concern due to the potential, observed in prior studies, of BZE to exhibit vasoconstrictive activity and to be more potent than cocaine in producing seizures when administered ventricularly in rats [204]. The increased brain BZE could arise from the effect of ethanol producing an alteration in distribution of benzoylecgonine into the CNS or from cocaine metabolism within the brain. To further evaluate this result, mice were injected with BZE (10mg/kg IP) with or without ethanol pretreatment. Ethanol pretreatment resulted in a 3-fold decrease in serum BZE 30 minutes after the BZE dose, and a slower decline in serum BZE concentrations. However, while BZE achieved significant concentrations in brain when administered alone, no BZE was detected with ethanol pretreatment. This would indicate ethanol does not increase distribution to the brain. In addition, due to its low lipophilicity, BZE would not be expected to cross the blood brain barrier to any significant degree. Further study will be required to explain this interesting finding.

In the mice studies already discussed, one significant question arises given the route of administration selected. Is the increased cocaine concentration observed due to a metabolic effect, due to ethanol effects on the membrane of the blood brain barrier allowing more cocaine to enter the brain, or due to an effect of

ethanol increasing the absorption of cocaine from the peritoneal cavity? To eliminate the absorption question, a fourth study was done using intravenous administration of cocaine. In this study, female ICR mice were administered a single dose of cocaine (5mg/kg IV) with or without ethanol pretreatment (2g/kg gavage). Ethanol pretreatment resulted in statistically significant increases in maternal blood cocaine concentration at 15 and 30 minutes after the cocaine dose. Blood BZE concentrations were significantly decreased. The changes in serum cocaine and benzoylecgonine concentrations produced by ethanol pretreatment are consistent with an inhibition of cocaine esteratic metabolism and support hypothesis 2 for this project. However, brain cocaine concentrations were not increased with ethanol pretreatment. In fact, there was a lower (although statistically insignificant, $p>0.05$) peak cocaine concentration achieved. No BZE was detected in the brain tissue of either group. Fetal brain cocaine concentrations were not significantly different with ethanol pretreatment. As discussed earlier, the apparent elimination rate and half-life of cocaine from brain tissue was calculated (Table 3-37). Statistically significant differences in elimination half-lives were observed between the cocaine only and cocaine/ethanol treatment groups for maternal brain cocaine. Fetal elimination half-lives were not significantly different between the groups. These results indicate that ethanol does not significantly alter cocaine distribution to the fetus and therefore do not support the hypothesis that ethanol/cocaine interactions would increase cocaine concentration in the fetus. While fetal brain cocaine concentrations are higher with ethanol pretreatment (Table 3-36) at 60 and

90 minutes, the observed increases are not statistically significant. Suggestions for future research would include analysis of the whole fetus rather than only the fetal brain. In addition, further study is warranted to examine possible male/female sex related differences in metabolism as well as pregnancy related differences. With pregnancy, cocaine is distributed to the placenta and amniotic cavity as well as to the fetal brain and body which could alter drug distribution.

Finally, cocaine and ethanol interactions were observed following acute (1 dose) and chronic (24 day) administration of cocaine (10mg/kg IP) with or without ethanol (1.5mg/kg gavage) in rats. As seen in Figures 3-19 and 3-20, ethanol treatment resulted in higher mean peak cocaine concentrations in blood. Ethanol treatment also resulted in a statistically significant increase in the blood cocaine AUC in the rats treated chronically as well as a higher mean peak cocaine concentration. In rats given acute doses, the time to reach peak cocaine concentrations was faster in the ethanol treated group and the mean peak cocaine concentration was significantly higher. It took significantly longer to reach peak BZE concentrations in the ethanol treated rats which indicates inhibited metabolism of cocaine to BZE. The changes in blood cocaine and benzoylecgonine concentrations produced by ethanol treatment are consistent with an inhibition of cocaine esteratic metabolism and support hypothesis 2 for this project.

The final aim of this project was to examine the effect of cocaine and its metabolites on T and B lymphocyte proliferation (*in vitro*). In addition to the toxic effects of cocaine outlined earlier, evidence exists to suggest that cocaine may have

a marked effect on the immune system. While some immunomodulatory effects of cocaine have been studied, no studies have examined the direct effect of any cocaine metabolites. In addition, most previous work has focused on the investigation of T-cell immunomodulation.

This study examines the effect of cocaine and metabolites on human B cell proliferation using IM-9 cells. This human B lymphoblastoid line is derived from a patient with multiple myeloma. IM-9 cells are capable of synthesizing IgG and IgA [205], and have been used extensively in biosynthetic and surface marker studies. They also possess receptors for human growth hormone, insulin, IGF-II, and substance P [206-207], as well as producing and secreting immunoreactive growth hormone and prolactin [208-209]. These cells also produce an autostimulatory growth factor which allows the cells to increase their own proliferation 2-3 fold in cells cultured at low density [210].

As mentioned in Chapter 1, several studies have investigated the effects of cocaine on human and animal immune functions. Studies evaluating the effect of cocaine on cellular immune function have indicated that the drug causes suppression of mitogenic responses in human and mouse T lymphocytes. More significantly, cocaine (at concentrations observed in human cocaine abusers) was demonstrated *in vitro* to augment human T-lymphocyte proliferation stimulated by the anti-CD3 antibody. This was designed to more accurately mimic the *in vivo* stimulation of T-cells via the T-cell receptor complex than is PHA-activation [140]. This study utilized a B cell line since present studies of humoral immunity have produced less definitive information on the immunomodulatory effects of cocaine.

A complicating factor of many published studies with cultured lymphocytes has been that the dose levels far exceed blood plasma levels found in human cocaine users. A dose response range of 0.1-10.0ug/ml was used in this study to better reflect typical blood cocaine concentrations. Within this concentration range, we have shown that cocaine and its major metabolites exert a stimulatory effect on the proliferation of IM-9 human B cells. Incubation with cocaine showed increased proliferation of 110-148% of the control response (Figure 3-21). The cocaine metabolites norcocaine, benzoylecgonine and cocaethylene were shown to have similar immunomodulatory effects on IM-9 cells as the parent compound. Incubation with benzoylecgonine showed increased proliferation of 109-170% of the control response, norcocaine 114-150%, and cocaethylene 100-146% (Figures 3-22 to 3-24). This implies that the immune consequences of cocaine use would therefore be significantly prolonged. While norcocaine and cocaethylene stimulate cell proliferation only in serum-free medium, cocaine and benzoylecgonine also stimulate proliferation in 2%FBS medium. This indicates that the proliferative responses may be occurring through different mechanisms and that cocaine and benzoylecgonine may have the potential to cause cell proliferation *in vivo* where the cells would be in the presence of serum.

The final study completed for this project was an examination of the proliferative response of lymphocytes isolated from the cord blood of neonates exposed to cocaine during gestation. Control lymphocytes were also isolated from the cord blood of neonates whose mothers had used no drugs during pregnancy.

Subjects were excluded from the study if, during pregnancy, they had: smoked marijuana, used illicit drugs other than cocaine, drank more than 1.5 ounces of ethanol per day, took immunosuppressive drugs or steroids, or if they had cancer or were HIV positive or had AIDS. Subjects were not excluded for smoking tobacco. The cord blood mononuclear cells were stimulated with the mitogen phytohemagglutinin (PHA). This mitogen was chosen because it stimulates the first signal transduction pathway in T cell activation and because it has been shown that fetal lymphocytes respond to this mitogen. The results indicate that the cocaine exposed target group showed increased (although this increase was not statistically significant) proliferation as compared to the control group. There was considerable variability in the responsiveness of PBMCs isolated from different individuals (Table 3-44). This increase would seem to suggest the cocaine targets mount a more aggressive immune response to the mitogenic challenge; however, until more sophisticated analyses are completed, whether this response is a positive or negative response cannot be determined. Recent reports [142-143] would suggest that a more significant indicator of the immunotoxicology of cocaine would be to perform cell population counting on the cord blood samples. This data would yield information on whether certain cell types are increased or decreased and what the implications of those findings may be (for example, decreased numbers of CD4+ cells and increased CD8+ cells). Support for this argument comes from a recent finding that while very low birthweight babies have normal numbers of T-cells and natural killer cells, they possess a less than optimal cellular functional capacity,

thereby leaving them vulnerable to infection [211]. In that study, T-lymphocyte subsets were analyzed from the peripheral blood of 6 VLBW infants on day 1-3 and on day 30. When compared to control newborns 4-5 days old, all had comparable percentages and total numbers of CD3+ T-cells (mature T-cells) and four had comparable percentages of CD8+ cells. In addition, these infants showed a 2-fold increase in the percentage of natural killer cells [211]. A suggestion for future research would be to enroll cocaine exposed newborns in a clinical study which follows them for the first year of life to evaluate if these infants have more infections or altered immune cell numbers as compared to non-cocaine exposed infants.

Lastly, interest in the immunotoxicology of cocaine has risen due to concern that cocaine abuse may, in some cases, be a possible co-factor in HIV transmission or disease progression. Results of this project, coupled with literature reports, support the hypothesis that cocaine has immunomodulatory action. Since a majority of perinatally acquired HIV infections are associated with IV drug use by the mother or her sexual partner, any effect of cocaine to compromise the development of the fetal immune system would have serious consequences. For example, vasculitis has been reported to be associated with cocaine use [153]. It is speculated that if this occurs as placentitis or chorioamnionitis (infections occurring during labor) in an HIV infected woman, the permeability of the placental barrier to maternal blood could be altered and HIV-infected inflammatory cells could accumulate at the maternal-fetal interface and more readily lead to neonatal HIV infection. More research is certainly warranted to provide more definitive answers

to the question of the effect of cocaine and metabolites on the developing fetal immune system.

Based on the results of the research for this study, the following conclusions can be made:

1. HPLC is a valid method for the analysis and quantitation for cocaine and its metabolites in a range of biological samples.

2. HPLC provides excellent reproducibility, sensitivity, and quantitation when compared to GC/MS. A major advantage of the analysis of samples by HPLC is that less sample handling is required as both benzoylecgonine and norcocaine must be derivatized prior to analysis by GC/MS.

3. The fetus has reduced capacity to metabolize cocaine to benzoylecgonine when compared to the adult.

4. Comprehensive meconium testing by chromatographic analysis provides significantly better incidence identification and test sensitivity than does urine immunoassay targeted to individuals presenting with specific risk factors. The protocols used by hospitals to "target" individuals for drug testing are inadequate. This finding brings into question previous reports comparing cocaine exposure to clinical effect.

5. The highest concentrations of cocaine metabolite (BZE) were found in amniotic fluid -- to which the fetus is continually exposed.

6. The fetus does not receive higher concentrations of cocaine than the mother initially following maternal cocaine use. However, concentrations remain higher in the fetus for a longer period of time due to slower elimination.

7. In mice, concurrent treatment with cocaine and ethanol resulted in a 5-fold increase in peak serum cocaine concentrations and as much as a 6-fold increase in peak brain cocaine concentrations. Peak cocaine concentration was reached faster in the ethanol treated rats and mice.

8. In mice, concurrent treatment with cocaine and ethanol resulted in significantly greater brain BZE concentrations and decreased serum concentrations.

9. Cocaine and its metabolites stimulate proliferation of IM-9 cells (human B-lymphoblastoid cells) in *in vitro* culture.

APPENDIX A
CALIBRATION CURVES

Sample Calibration for Benzoylecgonine by AD$_x$™

Calibration Level	Concentration (ng/ml)	Net Polarization	Net Fluorescence Intensity
A	0	197.88	3195
B	300	166.05	3332
C	1000	134.49	3472
D	2000	114.40	3536
E	3000	103.58	3589
F	5000	89.68	3628

Sample Calibration for Benzoylecgonine by HPLC

Concentration (ng/ml)	Response	Std.Dev.	N
100	530	10	2
250	1070	10	2
500	2350	0	2
750	2980	30	2
1000	4130	0	2
1250	5030	10	2
1500	6080	10	2
1750	6930	20	2

Sample Calibration for Norcocaine by HPLC

Concentration (ng/ml)	Response	Std.Dev.	N
100	380	40	2
250	820	70	2
500	1520	10	2
750	2350	20	2
1000	3190	10	2
1250	3950	30	2
1500	4800	0	2
1750	5620	50	2

Sample Calibration for Cocaethylene by HPLC

Concentration (ng/ml)	Response	Std.Dev.	N
250	550	40	2
500	1250	10	2
750	1520	60	2
1000	2090	30	2
1250	2620	10	2
1500	3140	40	2
1750	3610	0	2

APPENDIX B
INTERDAY AND INTRADAY VARIABILITY RAW DATA

System 1 Interday Variability for 1000ng/ml

BZE		Cocaine		Norcocaine		Cocaethylene	
Conc.	Date	Conc.	Date	Conc.	Date	Conc.	Date
1002	6/3/93	1059	5/26/93	1276	6/3/93	1293	6/3/93
1053	6/1/93	1278	6/3/93	987	6/1/93	923	6/19/93
1226	6/19/93	1016	6/1/93	874	6/19/93	1046	6/24/93
991	6/24/93	949	6/19/93	1004	6/24/93	966	6/27/93
957	6/27/93	1045	6/24/93	1021	6/27/93	989	7/1/93
1091	7/1/93	1028	6/27/93	1040	7/20/93	800	7/20/93
1017	7/20/93	1046	7/1/93	1004	7/22/93	1041	7/22/93
848	7/22/93	992	7/20/93	1091	12/1/93	981	12/1/93
1113	12/1/93	941	7/22/93				
		1085	12/1/93				

System 2 Interday Variability for 1000ng/ml

BZE		Cocaine		Norcocaine		Cocaethylene	
Conc.	Date	Conc.	Date	Conc	Date	Conc.	Date
1003	10/9/93	908	10/9/93	1078	10/9/93	982	10/9/93
1004	4/19/94	985	4/19/94	979	4/19/94	926	4/19/94
984	4/19/94	974	4/19/94	983	4/19/94	962	4/19/94
1009	2/9/94	1021	2/9/94	921	2/9/94	977	2/9/94
1056	8/11/93	955	8/11/93	1076	8/11/93	1215	8/11/93
1062	8/11/93	952	8/11/93	1087	8/11/93	1221	8/11/93
1031	12/20/93	1016	12/20/93	1019	12/20/93	1032	12/20/93
985	12/20/93	1017	12/20/93	1009	12/20/93	1018	12/20/93
1014	8/18/93	934	8/18/93	1107	8/18/93	992	8/18/93
1029		947		1114		902	
1016		947		1109		913	
1010		958		1118		932	
953	10/27/94	976	10/27/94	961	10/27/94	814	10/27/94

System 2 Intraday Variability for 1000ng/ml

Date	BZE	Cocaine	Norcocaine	Cocaethylene
10/9/93	1003	908	1078	982
	997	897	1009	944
	975	897	1067	944
	999	990	1099	952
	999	872	1088	952
	1002	883	1088	944
	977	897	1009	948
	1060	898	1026	951
	996	933	1038	1016
	983	939	1037	1022
	993	930	994	971

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BIOGRAPHICAL SKETCH

Diane Lynne Phillips was born June 24, 1966, in Cincinnati, Ohio. She moved to Alaska with her family in 1981 where she graduated from Kenai Central High School, Kenai, Alaska, in 1984. Diane graduated from the University of Alaska Fairbanks in May 1988 with a B.S. in chemistry and from the University of Illinois at Chicago in June 1990 with a M.S. in forensic toxicology. She then returned to Alaska to engage in full-time employment in order to earn enough money to repay her student loans. In January 1992, on completion of that goal, Diane returned to graduate school at the University of Illinois to study for a Ph.D. in pharmaceutical sciences/toxicology. She transferred to the University of Florida in 1993 to complete her studies when her research advisor accepted a position at UF. While at the University of Florida, she received several college and national honors for her research, including a national award from the American Association of Pharmaceutical Scientists (AAPS) for outstanding research where she presented her work in the 1994 Eli Lilly Graduate Student Symposium in Pharmacokinetics, Pharmacodynamics, and Clinical Sciences (San Diego, CA); Rho Chi pharmacy honor society; and Phi Kappa Phi honor society. Diane intends to pursue a career in pharmaceutical research after graduation May 6, 1995.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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